Granted claims in Australia
(AU 742 818)

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A complex between at least one (negatively charged) nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature and the polymeric conjugate containing a polymer formed from the monomer units carrying free NH_3^+ functions, and being such that:

- the free NH_3^+ functions of the monomer units are substituted in a ratio of at least 10% by residues capable of being protonated in a weakly acid medium causing destabilization of cell membranes, in particular the cell membrane of endocytosis vesicles, and/or of endosomes, wherein,

- the residues have the following properties:

. they carry a functional group which enables them to be bonded to the polymer;

. they are not active with respect to a recognition signal recognized by a cell membrane receptor; and

. they can carry at least one free NH_3^+ function;

with the proviso that free NH_3^+ functions make up at least 30% of the number of monomer units of the polymer.

2. A complex accordingly to claim 1 wherein free NH_3^+ functions of the monomer units are substituted by non-charged residues causing a reduction in the positive charges of the polymeric conjugate, facilitating salting out of the nucleic acid in the course of dissociation of the complex.

3. A complex according to claim 2 wherein the non-charged said residues have the following properties:

- they carry at least one hydroxyl group; and

- they are not active with respect to the recognition signal recognized by a cell membrane receptor.

4. A complex according to any one of claims 1 to 3 further comprising molecules constituting a recognition signal recognized by a cell membrane receptor being present:

- by substitution of some of the free NH_3^+ functions of the monomer units, or

- on some of the non-charged residues causing a reduction in the charge in particular on hydroxyl groups of the non-charged residues, or

- on some of the residues causing a destabilization of cell membranes, or

- by substitution of the free NH_3^+ function of the residues causing a destabilization of cell membranes.

5. A complex according to any one of claims 1 to 4 wherein the monomer units are substituted in a ratio of from about 15% to about 45% by said residues.

6. A complex according to claim 5 wherein the monomer units are substituted in a ratio of 35% by said residues.

7. A complex between at least one (negatively charged) nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying free NH_3^+ functions, and being such that:

- the free NH_3^+ functions of the monomer units are substituted in a ratio of at least 10% by residues capable of being protonated in a weakly acid medium causing

destabilization of cell membranes, in particular the cell membrane of endocytosis vesicles and/or of endosomes, wherein:

the residues have the following properties:

. they are bases of which the pK in an aqueous medium is less than 8, such that a proportion greater than 50% of these bases bonded to the polymer is not protonated in a neutral medium of pH 7.4,

. they carry a functional group which enables them to be bonded to the polymer,

. they are not active with respect to a recognition signal recognized by a cell membrane receptor, and

. they can carry at least one free NH_3^+ function;

with the proviso that all the free NH3+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

8. A complex according to claim 7 wherein the free NH_3^+ functions of the monomer units are substituted by non-charged residues causing a reduction in the positive charges of the polymeric conjugate, facilitating salting out of the nucleic acid in the course of dissociation of the complex.

9. A complex according to claim 8 wherein the non-charged said residues have the following properties:

- they carry at least one hydroxyl group; and

- they are not active with respect to the recognition signal recognized by a cell membrane receptor.

10. A complex according to any one of claims 7 to 9 further comprising molecules constituting a recognition signal recognized by a cell membrane receptor being present:

- by substitution of some of the free NH_3^+ functions of the monomer units,

- on some of the non-charged residues causing a reduction in the charge, and in particular on hydroxyl groups of the non-charged residues causing a reduction in charge, or

- on some of the residues causing a destabilization of cell membranes; or

- by substitution of the free NH_3^+ function of the residues causing a destabilization of cell membranes.

11. A complex according to any one of claims 7 to 10 wherein the monomer units are substituted in a ratio of from about 15% to about 45% by said residues.

12. A complex according to claim 11 wherein the monomer units are substituted in a ratio of 35% by said residues.

13. A complex between at least one (negatively charged) nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature and the polymeric conjugate containing a polymer formed from monomer unites carrying free NH_3^+ functions, and being such that:

- the free NH_3^+ functions of the monomer units are substituted in a ratio of at least 10% by residues capable of being protonated in a weakly acid medium causing destabilization of cell membranes, in particular the cell membrane of endocytosis vesicles, wherein,

- residues belong to one or more families of compounds selected from:

. the family of compounds which carry an imidazole nucleus,

. the family of quinolines,

. the family of pterines, and

. the family of pyridines, and

wherein the residues have the following properties:

. the residues carry a functional group which enables them to be bonded to the polymer,

. they can carry at least one free NH_3^+ function; and

. they are not active with respect to a recognition signal recognized by a cell membrane receptor;

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymer.

14. A complex according to claim 13 wherein the free NH_3^+ functions of the monomer units are substituted by at least one molecule which constitutes a recognition signal recognized by a cell membrane receptor, and/or by non-charged residues causing a reduction in the positive charges of the polymeric conjugate, facilitating salting out of the nucleic acid in the course of dissociation of the complex.

15. A complex according to claim 13 wherein the free NH_3^+ functions of the monomer units are substituted by at least one molecule which constitutes a recognition signal recognized by a cell membrane receptor, and/or by non-charged residues causing a reduction in the positive charges of the polymeric conjugate, facilitating salting out of the nucleic acid by dissociation of the complex.

16. A complex according to claim 15 wherein the non-charged said residues have the following properties:

- they carry at least one hydroxyl group,

- they are not active with respect to the recognition signal recognized by a cell membrane receptor.

17. A complex according to claim 13 further comprising molecules constituting a recognition signal recognized by cell membrane receptors being present:

- by substitution of some of the free NH_3^+ functions of the abovementioned monomer units, or

- on some of the non-charged residues, and in particular on hydroxyl groups of the non-charged residues causing a reduction in charge, or

- on some of the residues causing a destabilization of cell membranes, or

- by substitution of the free NH_3^+ function of the residues causing a destabilization of cell membranes.

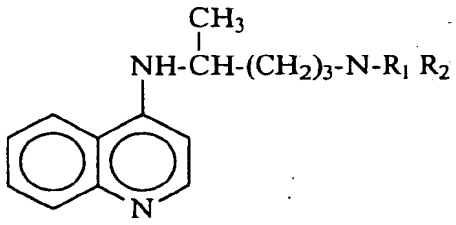
18. A complex according to any one of claims 13 to 17 wherein the monomer units are substituted in a ratio of from about 15% to about 45% by said residues.

19. A complex according to claim 18 wherein the monomer units are substituted in a ratio of 35% by said residues.

20. A complex according to any one of claims 1 to 19, in which the residues for causing destabilization of cell membranes in a weakly acid medium are:

- alkylimidazoles in which the alkyl radical contains 1 to 10 carbon atoms, and in which only one of the nitrogen atoms of the imidazole nucleus is substituted,

- or quinolines of the formula:



in which R_1 represents H and R_2 represents $(CH_2)_n$-CO_2-H, n being an integer varying from 1 to 10, and preferably having a value of 1 to 3.

21. A complex according to claim 20, wherein the alkyl radicals contain from 2 to 6 carbon atoms.

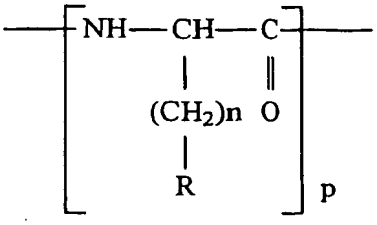
22. A complex according to claim 20 or 21 wherein the free NH_3^+ function of the residues is substituted by a molecule which constitutes a recognition signal recognized by a cell membrane receptor, with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer unites of the polymer.

23. A complex according to claim 22 wherein the residues for causing destabilization of cell membranes are selected from: histidine, 4-carboxymethyl-imidazole, 3-(1-methyl-imidazol-4-yl)-alanine, 3-(3-methyl-imidazol-4-yl)-alanine, 2-carboxy-imidazole, histamine, 3-(imidazol-4-yl)-L-lactic acid, 2-(1-methyl-imidazol-4-yl)ethylamine, 2-(3-methyl-imidazol-4-yl)ethylamine, β-alanyl-histidine-(carnosine), 7-chloro-4-(amino-1-methylbutylamino)-quinoline, N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine, 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline (primaquine), N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine, quininic acid, quinolinecarboxylic acid, pteroic acid, nicotinic acid and quinolinic acid.

24. A complex according to one of the claims 1 to 23 wherein remaining said free NH_3^+ functions of the monomer units are substituted to an extent of from about 1% to about 60% by a molecule which constitutes a recognition signal recognized by a cell membrane receptor, this recognition signal having a molecular weight less than 5,000.

25. A complex according to claim 24 wherein the recognition signal is present in an amount of from one molecule per 200 monomer units of polymeric conjugate to about 60 molecules per 200 monomer units, with the proviso that the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymer.

26. A complex according to one of claims 1 to 20, in which the polymer comprises a polymer of formula (I):



(I)

wherein:

- p is an integer of from 15 to 900,

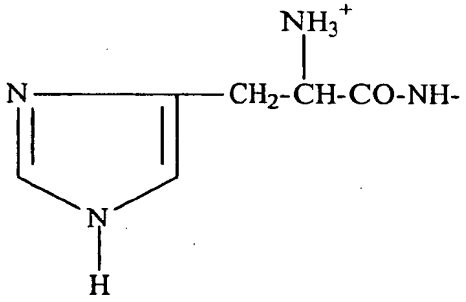
- n is an integer varying from 1 to 6,

and contains radicals R wherein:

- 10% to 45% of the number of radicals R comprise a residue with an imidazole nucleus.

27. A complex according to claim 26 wherein the residue with the imidazole nucleus has a free NH_3^+ function.

28. A complex according to claim 26 or 27 wherein the residue with the imidazole nucleus comprises:



29. A complex according to any one of claims 26 to 28, wherein the NH_3^+ function of the residue with the imidazol nucleus is substituted by a molecule which constitutes a recognition signal.

30. A complex according to any one of claims 26 to 28, wherein 10% to 90% of the number of radicals R represent free ϖ-amino NH_3^+.

31. A complex according to claim 30 wherein the free ϖ-amino NH_3^+ is substituted to an extent of 0 to 50% by a molecule which constitutes a recognition signal recognised by a cell membrane receptor.

32. A complex according to claim 30 wherein the free ϖ-amino NH_3^+ is substituted to an extent of from 0 to 60% by a molecule which constitutes a recognition signal.

33. A complex according to claim 30 wherein the free ϖ-amino NH3+ is substituted to an extent of 1 said molecule per 200 monomer units.

34. A complex according to claim 30 wherein the free ϖ-amino NH_3^+ is substituted to an extent of from 2 to 100 said molecules per 200 monomer units.

35. A complex according to claim 34 wherein the free ϖ-amino NH_3^+ is substituted to an extent of about 50 said molecules per 200 monomer units.

36. A complex according to claim 26 wherein the radicals R comprise from 0 to 45% of a group of formula $NH\text{-}CO\text{-}(CHOH)_m\text{-}R_1$.

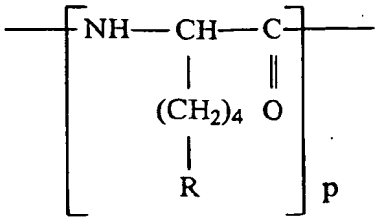
37. A complex according to claim 36 wherein said group is dihydroxypropionylamido, erythronylamido, threonylamido, ribonylamido, arabinylamido, xylonylamido, lyxonylamido, gluconylamido, galactonylamido, mannonylamido, glycoheptonylamido or a glycooctonylamido radical, m is an integer from 2 to 15, R_1 represents H or an alkyl radical having 1 to 15 carbon atoms.

38. A complex according to claim 37 wherein m is an integer of from 2 to 7.

39. A complex according to claim 37 or 38 wherein R_1 represent CH_3.

40. A complex according to claim any one of claims 37 to 39 wherein at least some of the groups of formula $NH\text{-}CO\text{-}(CHOH)_m\text{-}R_1$ are substituted by a molecule which constitutes a recognition signal recognised by a cell membrane receptor.

41. A complex according to any one of claims 1 to 19, in which the polymer comprises a polymer of the following formula (II):



(II)

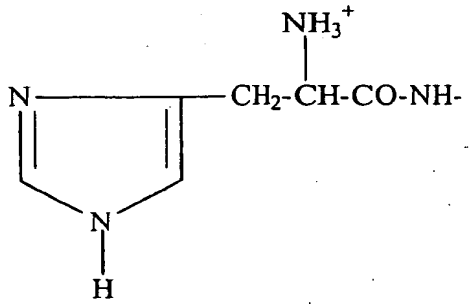
wherein:

- p is an integer of from 15 to 900 and R represents radicals R wherein:

- 10% to 45% of the number of radicals R represent a residue carrying an imidazole nucleus.

42. A complex according to claim 41 wherein the residue with the imidazole nucleus has a free NH_3^+ function.

43. A complex according to claim 41 or 42 wherein the residue with the imidazole nucleus comprises:



44. A complex according to any one of claims 41 to 43 wherein the NH_3^+ function at least some of the residues with the imidazole nucleus are substituted by a molecule which constitutes a recognition signal for a cell receptor.

45. A complex according to any one of claims 41 to 44 wherein:

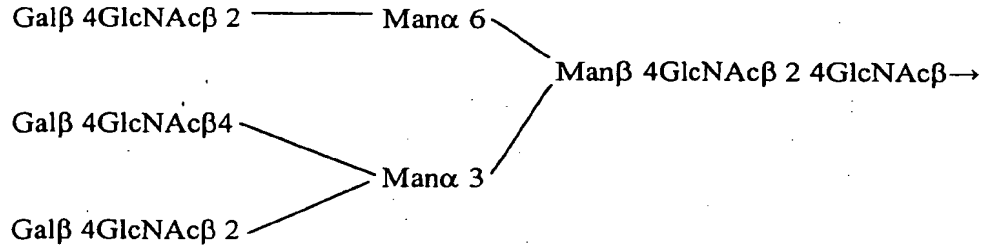
- the remainder of the radicals R represent ϖ-amino NH_3^+.

46. A complex according to claim 41 or 42 wherein from 0 to 45% of the radicals R are substituted by a molecule which constitutes a recognition signal recognized by a cell membrane receptor.

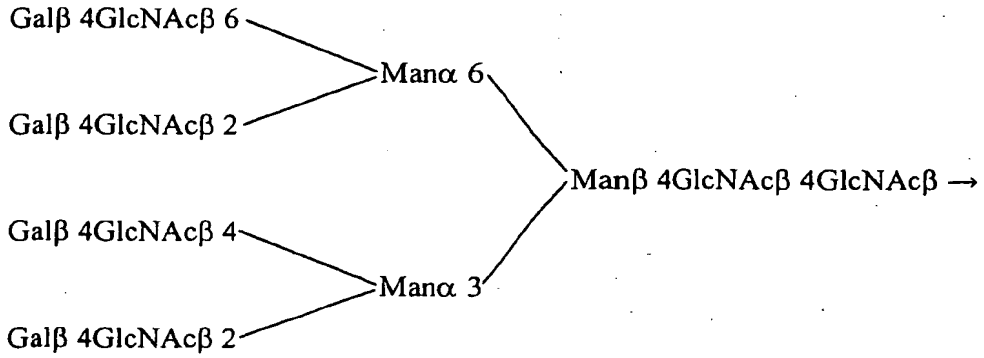
47. A complex according to one of claim 1 to 19 wherein the recognition signal is selected from:

A. a) simple or complex osides recognized by membrane lectins and chosen from:

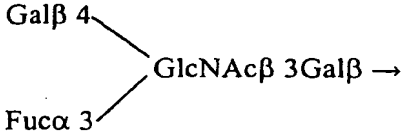
a.Asialo-oligoside of the type of triantennar lactosamine: asialoglycoprotein receptor



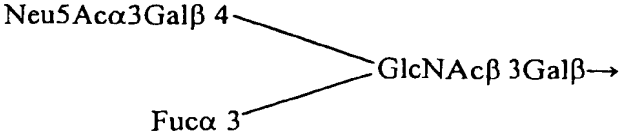
b) Asialo-oligoside of the type of tetraantennar lactosamino: asialoglycoportien receptor



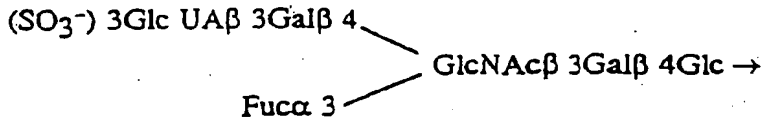
c) Lewis x: LECAM 2.3



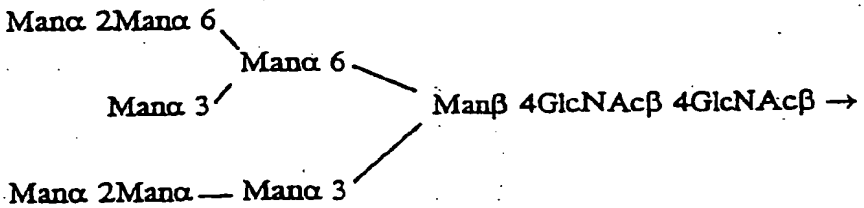
d) Lewis x sialyl: LECAM 3/2



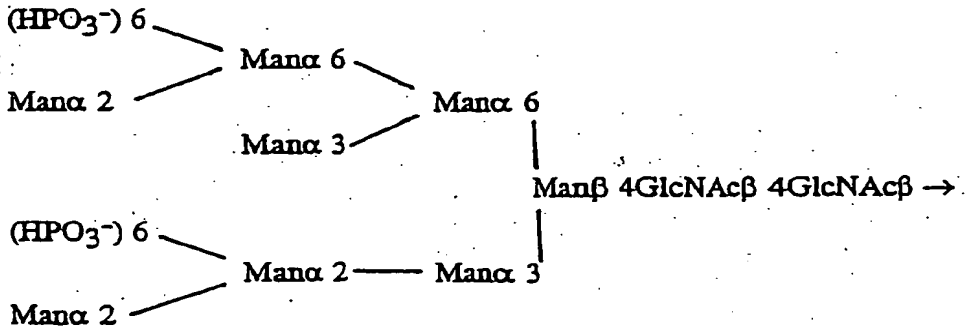
e) Sulphated Lewis x derivative (HNK1): LECAM 1



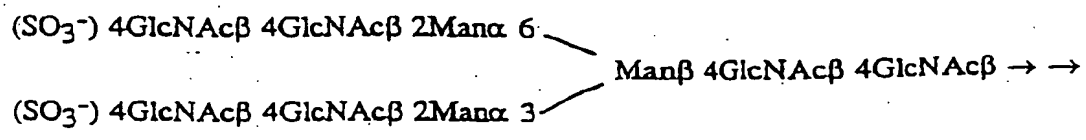
f) Oligomannoside: mannose receptor



g) Phosphorylated oligomannoside: mannose 6-phosphate receptor



h) Oligosaccharide of the type of sulphated lactosamine: sulphated GalNAc 4 receptor



B) Peptides

a) anti-inflammatory peptides of fragments thereof, recognized by receptors of the vascular wall,

b) ligand peptides of integrins,

c) chemiotactic factors,

d) peptide hormones, and

C. natural metabolites.

48. A complex according to claim 47 wherein the anti-inflammatory peptides are selected from:

- vasodilator intestinal polypeptide (VIP)

HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2,

- atrial natriuretic polypeptide (ANP)

SLRRSSCFGGRMDRIGAQSGLGCNSFRY,

- lipocortin

HDMNKVLDL, and

- bradykinin

RPPGFSPFR.

49. A complex according to claim 47 wherein the ligand peptides are selected from peptides containing the sequence RGD or fibronectin ligand.

50. A complex according to claim 47wherein the chemotactic factors are selected from formyl-peptides and their antagonists, FMLP and (N-formyl-Met-Leu-Phe).

51. A complex according to claim 47 wherein the peptide hormones are selected from α-MSH, Ac-SYSMEHFRWGKPV-NH_2 and their antagonists.

52. A complex according to claim 47 wherein the natural metabolites are selected from biotin, carnitine, tetrahydrofolate and folic acid.

53. A complex according to claim 47 wherein the natural metabolites are both a recognition signal for cells having suitable receptors and a destabiliser of cell membranes.

54. A complex according to any one of claims 1 to 53 wherein the nucleic acid is selected from the group consisting of:

a) marker genes,

b) genes with therapeutic purpose, and

c) genes for the purpose of vaccines.

55. A complex according to claim 54 wherein the marker genes comprise genes coding for luciferase, green protein of the jellyfish Aequarea Victoria, β-galactosidase, chloramphenicol acetyltransferase and genes which confer resistance to antibiotics.

56. A complex according to claim 54 wherein the genes with a therapeutic purpose are selected from genes coding for :

- receptors of lipoproteins of low-density, which are deficient in cases of hypercholesterolaemia,
- coagulation factors: factors VIII and IX,
- phenylalanine hydroxylase (phenylketonuria),
- adenosine deaminase (ADA immunodeficiency),
- lysosomal enzymes, such as β-glucosidase in the case of Gaucher's disease,
- dystrophin and minidistriphin (myopathy),
- tyrosine hydroxylase (Parkinson),
- neurone growth factors (Alzheimer),
- CFTR cystic fibrosis transmembrane conductance regulator (cystic fibrosis),
- alpha-1-antitrypsin,
- cytokines (interleukins, TNF tumour necrosing factor),

- thymidine kinase of the Herpes simplex virus,

- proteins of major histocompatibility complex MHC,

- cytosine deaminase,

- genes which code for sense and antisense RNAs, and

- genes which code for ribozymes.

57. A complex according to claim 54 wherein the genes for the purpose of vaccines are selected from genes which code for viral antigens and the gene which codes for the nucleoprotein of the influenza virus.

58. A complex according to any one of claims 1 to 40 wherein:

- the polymer comprises polylysine having a degree of polymerisation of from about 15 to about 900,

- the free NH_3^+ functions of the lysine monomer units are substituted in a ratio of 35% by histidyl residues,

- the nucleic acid has a molecular weight of from about 10^6 to about 10^8, and

- the ratio between the number of base pairs of the nucleic acid per lysine is from about 0.2 to about 6.

59. A complex according to claim 58 wherein the lysine monomers are substituted by a molecule which constitutes a recognition signal in a ratio of from 1 said molecule to 50 residues of lysine, wherein the molecule has an affinity of at least 10^5 1 $mole^{-1}$ for the receptor of the cell which the complex is to target.

60. A complex according to claim 59 wherein the lysine monomers are substituted by the molecule in a ratio of 20 to 100 said molecules per 200 lysine monomers.

61. A complex according to any one of claims 58 to 60 wherein the nucleic acid has a molecular weight of from about $1x10^6$ to about $1x10^8$, and preferably from about 3×10^6 to 30×10^6.

62. A complex according to any one of claims 58 to 61 wherein the ratio between the average number of base pairs of the nucleic acid per lysine is from about 0.2 to about 6 and preferably, 0.4 to 0.6.

63. A positively charged polymeric conjugate containing units carrying free NH_3^+ functions, wherein:

- the free NH_3^+ functions of the monomer unites are substituted in a ratio of at least 10% by residues for causing destabilization of cell membranes, in particular the membrane of endocytosis vesicles, in a weakly acid medium,

- the residues also having the following properties:

. they carry a functional group which enables them to be bonded to the abovementioned polymer,

. they are not active with respect to a recognition signal recognized by a cell membrane receptor, and

. they can carry at least one free NH_3^+ function.

- at least some of the free NH_3^+ functions of the monomer units being substituted by non-charged residues causing a reduction in the positive charges of the polymeric conjugate, facilitating salting out of the nucleic acid by dissociation of the complex, wherein the non-charged residues have the following properties:

- they carry at least one hydroxyl group,

- they are not active with respect to a recognition signal recognized by the cell membrane receptor, and

- the hydroxyl groups of the at least some of the non-charged residues are substituted by at least one molecule which constituted a recognition signal recognized by a cell membrane receptor,

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymer.

64. A complex according to claim 63 further comprising molecules constituting a recognition signal recognized by a cell membrane receptor being present:

- by substitution of some of the free NH_3^+ functions of the monomer units, or

- on some of the abovementioned non-charged residues causing a reduction in the charge, on the hydroxyl groups of the non-charged residues, or

- on some of the residues causing a destabilization of cell membranes, or

- by substitution of the optional free NH_3^+ function of the residues causing a destabilization of cell membranes.

65. A polymeric conjugate according to claim 63 or 64 comprising a complex between the positively charged polymeric conjugate and at least one (negatively charged) nucleic acid, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature, and wherein:

- the residues for causing destabilization of cell membranes, in particular the cell membrane of endocytosis vesicles and/or of endosomes are capable of being protonated in a weakly acid medium; and

. they have a pK in an aqueous medium less than 8, such that a proportion greater than 50% of these bases bonded to the polymer is not protonated in a neutral medium of pH 7.4.

66. A polymeric conjugate according to claim 65 wherein the residues capable of being protonated belong to one or more families of compounds selected from:

. the family of compounds which carry an imidazole nucleus,

. the family of quinolines,

. the family of pterines, and

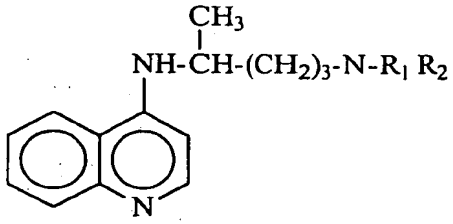
. the family of pyridines, and

wherein at least some of the residues carry at least one free NH_3^+ function.

67. A polymeric conjugate according to claim 65 wherein said residues capable of being protonated in a weakly acid medium are:

- alkylimidazoles in which the alkyl radical contains 1 to 10 carbon atoms, and in which only one of the nitrogen atoms of the imidazole nucleus is substituted,

- or quinolines of the formula:



in which R_1 represents H and R_2 represents $(CH_2)_n$-CO_2-H, n being an integer varying from 1 to 10, and preferably having a value of 1 to 3.

68. A polymeric conjugate according to claim 67 wherein the alkyl radicals contain from 2 to 6 carbon atoms.

69. A polymeric conjugate according to claim 65 the residues capable of being protonated in a weakly acidic medium are selected from: histidine, 4-carboxymethyl-imidazole, 3-(1-methyl-imidazol-4-yl)-alanine, 3-(3-methyl-imidazol-4-yl)-alanine, 2-carboxy-imidazole, histamine, 3-(imidazol-4-yl)-L-lactic acid, 2-(1-methyl-imidazol-4-yl)ethylamine, 2-(3-methyl-imidazol-4-yl)ethylamine, β-alanyl-histidine-(carnosine), 7-chloro-4-(amino-1-methylbutylamino)-quinoline, N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine, 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline (primaquine), N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine, quininic acid, quinolinecarboxylic acid, pteroic acid, nicotinic acid and quinolinic acid.

70. Use of a complex according to one of claims 1 to 62 or a conjugate according to one of claims 63 to 69 for the *in vitro*, *ex vivo* or *in vivo* transfection of cell.

71. Use according to claim 68, wherein the cells are chosen from:

- cells of haematopoietic strains,
- dendritic cells,
- liver cells,
- skeletal muscle cells,
- skin cells:
  - . fibroblasts,
  - . keratinocytes,
  - . dendritic cells,
  - . melanocytes,
- cells of the vascular walls,
  - . endothelial,
  - . smooth muscle,
- epithelial cells of the respiratory tract,
- cells of the central nervous system,
- cancerous cells, and
- cells of the immune system, such a lymphocytes, macrophages, and NK cells.

72 . Method of *in vitro* or *ex vivo* transfection, comprising bringing a complex as defined in any one claims 1 to 62 into contact with a medium containing cells to be transfected under conditions such that there is:

- passage of the complex from the medium into the cytoplasm of the cells,
- salting out of the nucleic acid from the complex in the cytosol and/or the nucleus of the cells,

- transcription and expression of the nucleic acid in the transfected cells, and

- expression of the protein corresponding to the transfected gene.

73. A pharmaceutical composition comprising a complex as defined in any one of claims 1 to 62, or a conjugate as defined in any one of claims 63 to 69, in combination with a pharmaceutically acceptable vehicle.

74. Use of a complex as defined in any one of claims 1 to 62 or a conjugate as defined in any one of claims 62 to 69 in the preparation of a medicament intended for treatment of a congenital or acquired metabolic deficiency, or treatment of a tumour, or comprising a vaccine.

75. A kit comprising:

- a polymeric conjugate according to one of claims 63 to 69, and

- reagents for facilitating transfection of a cell by the conjugate.

76. A kit according to claim 75 further comprising a plasmid containing at least one gene to be transferred into the cell by the conjugate.

77. A kit according to claim 75 or 76 further comprising reagents for facilitating bonding of the recognition signal to the polymeric conjugate.

78. A kit according to any one of claims 75 to 77 further comprising reagents for facilitating the formation of a complex as defined in any one of claims 1 to 62, or between the polymeric conjugate and the gene to be transferred, or between the polymeric conjugate and a plasmid containing the gene to be transferred.

79. A complex formed by at least one negatively charged nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being ionic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying NH_3^+ groups, substantially as hereinbefore described.

80. A positively charged polymeric conjugate containing a polymer formed from monomer units a proportion of which carry residues for causing destabilisation of cell membranes, substantially as hereinbefore described.

81. Use of a complex in the *in vitro*, *ex vivo* or *in vivo* transfection of a cell, wherein the complex comprises at least one negatively charged nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being ionic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying NH_3^+ groups, substantially as hereinbefore described.

82. Use of a positively charged conjugate containing a polymer formed from monomer units a proportion of which carry residues for causing destabilisation of cell membranes, in the transfection of a cell, substantially as hereinbefore described.

83. A method of *in vitro* or *ex vivo* transfection of a cell involving contacting a cell with a complex formed by at least one negatively charged nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being ionic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying NH_3^+ groups, substantially as hereinbefore described.

84. A pharmaceutical composition comprising a pharmaceutically acceptable vehicle and a complex formed by at lest one negatively charged nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being ionic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying NH_3^+ groups, substantially as hereinbefore described.

85. A pharmaceutical composition comprising a pharmaceutically acceptable vehicle and a conjugate containing a polymer formed from monomer units a proportion of which carry residues for causing destabilisation of cell membranes, substantially as hereinbefore described.

86. Use of a complex in the manufacture of a medicament or preparation of a vaccine, wherein the complex is formed by at least one negatively charged nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being ionic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying NH_3^+ groups, substantially as hereinbefore described.

87. Use of a positively charged conjugate containing a polymer formed from monomer units a proportion of which carry residues for causing destabilisation of cell membranes, in the manufacture of a medicament or preparation of a vaccine, substantially as hereinbefore described.

DATED this 9th day of November 2001

I.D.M. IMMUNO-DESIGNED MOLECULES

Attorney: DAVID A. ADAMTHWAITE
Fellow Institute of Patent and Trade Mark Attorneys of Australia
of BALDWIN SHELSTON WATERS

Granted claims in Europe

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 946 744 B1

(12) FASCICULE DE BREVET EUROPEEN

(45) Date de publication et mention de la délivrance du brevet:
18.08.2004 Bulletin 2004/34

(21) Numéro de dépôt: **97945903.9**

(22) Date de dépôt: **10.11.1997**

(51) Int Cl.[7]: **C12N 15/88**, A61K 48/00

(86) Numéro de dépôt international:
PCT/FR1997/002022

(87) Numéro de publication internationale:
WO 1998/022610 (28.05.1998 Gazette 1998/21)

(54) NOUVEAUX COMPLEXES POLYMERIQUES POUR LA TRANSFECTION D'ACIDES NUCLEIQUES, AVEC DES RESIDUS DESTABILISANT DES MEMBRANES CELLULAIRES

NEUE POLYMERISCHE KOMPLEXE FÜR DIE TRANSFEKTION VON NUKLEINSAÜREN, MIT RESTEN FÜR DESTABILISIERUNG DER ZELLMEMBRANEN

NOVEL POLYMERIC COMPLEXES FOR THE TRANSFECTION OF NUCLEIC ACIDS, WITH RESIDUES CAUSING THE DESTABILISATION OF CELL MEMBRANES

(84) Etats contractants désignés:
AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priorité: **15.11.1996 FR 9613990**

(43) Date de publication de la demande:
06.10.1999 Bulletin 1999/40

(73) Titulaire: **I.D.M. IMMUNO-DESIGNED MOLECULES**
75011 Paris (FR)

(72) Inventeurs:
- **MIDOUX, Patrick**
  F-45100 Orléans (FR)
- **MONSIGNY, Michel**
  F-45590 Saint-Cyr-en-Val (FR)

(74) Mandataire: **Grosset-Fournier, Chantal Catherine**
Grosset-Fournier & Demachy,
54, rue Saint-Lazare
75009 Paris (FR)

(56) Documents cités:

EP-A- 0 350 246
EP-A- 0 387 775
EP-A- 0 388 758
WO-A-92/11037
WO-A-92/13570
FR-A- 2 107 756
FR-A- 2 719 316
US-A- 5 166 320

- **MEZÖ G. ET AL.: "Carrier design:conformational studies of amino acid (X) and oligopeptide (X-DL-Ala-m) substituted poly(L-lysine)." BIOPOLYMERS, vol. 33, no. 6, 1993, pages 873-885, XP002034603**
- **WANG C. Y. ET AL: "POLYHISTIDINE MEDIATES AN ACID-DEPENDENT FUSION OF NEGATIVELY CHARGED LIPOSOMES" BIOCHEMISTRY, vol. 23, no. 19, 1984, pages 4409-4416, XP002016041 cité dans la demande**
- **MIDOUX P ET AL: "SPECIFIC GENE TRANSFER MEDIATED BY LACTOSYLATED POLY-L-LYSINE INTO HEPATOMA CELLS" NUCLEIC ACIDS RESEARCH, vol. 21, no. 4, 25 février 1993, pages 871-878, XP000371764 cité dans la demande**
- **SANTELLA M. AND LI H.J.: 'Interaction between poly(L-Lysine, L-Histidine) and DNA' BIOPOLYMERS vol. 16, 1997, pages 1879 - 1894**
- **ROUFAI M.B. AND MIDOUX P.: 'Histidylated polylysine as DNA vector: Elevation of the imidazole protonation and reduced cellular uptake without change in the polyfection efficiency of serum stabilized negative polyplexes' BIOCONJUGATE CHEMISTRY vol. 12, no. 1, pages 92 - 99**

EP 0 946 744 B1

Printed by Jouve, 75001 PARIS (FR)

- Zellen des zentralen Nervensystems,
- Krebszellen,
- Zellen des Immunsystems.

22. Verfahren für die Transfektion *in vitro* oder *ex vivo*, **dadurch gekennzeichnet, dass** es in der Anwesenheit eines Komplexes nach einem der Ansprüche 1 bis 19 durchgeführt wird, in einem Medium, welches zu transfizierende Zellen unter Bedingungen enthält, die wie folgt sind:

- Passage des Komplexes vom Medium in das Zytoplasma der Zellen,
- Befreien der in dem vorher erwähnten Komplex enthaltenden Nukleinsäure im Cytosol und/oder im Kern der Zellen,
- Transkription und Expression der Nukleinsäure in den transfizierten Zellen,
- Expression der zum transfizierten Gen korrespondierenden Protein.

23. Pharmazeutische Zusammensetzung, **dadurch gekennzeichnet, dass** es als Wirkstoff wenigstens einen der Komplexe nach einem der Ansprüche 1 bis 19 zusammen mit einem pharmazeutisch akzeptablen Träger enthält.

24. Verwendung eines Komplexes nach einem der Ansprüche 1 bis 19 für die Herstellung eines Medikaments, beabsichtigt z.B. für die Behandlung vererbter oder erworbener metabolischer Störungen oder zur Behandlung von Tumoren oder zur Herstellung einer Vakzine, z.B. eine Vakzine gegen die Grippe.

Claims

1. Complex between at least one (negatively charged) nucleic acid and at least one positively charged polymeric conjugate, the bond between the nucleic acid and the polymeric conjugate being electrostatic in nature and the polymeric conjugate containing a polymer formed from monomer units carrying free NH_3^+ functions, and being such that:

   - the free NH_3^+ functions of the abovementioned monomer units are substituted in a ratio of at least 10% by residues which can be protonated in a medium, the pH of which is less than that of plasma or serum, said residues causing destabilization of cell membranes in a medium, the pH of which is less than that of plasma or serum,
   - the abovementioned residues also presenting the following properties:

     . they carry a functional group which enables them to be bound to the abovementioned polymer,
     . they are not active with respect to the recognition signal recognized by a cell membrane receptor,
     . they belong to the family of compounds which carry an imidazole nucleus,
     . they belong to the family of quinolines,
     . they belong to the family of pterines,
     . they belong to the family of pyridines,

   with the proviso that the totality of the free NH_3^+ functions is of at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate,

   and with the proviso that the polymeric conjugate is different from the polylysine-(dithiopyridyl)propionate conjugate.

2. Complex according to claim 1, wherein the residues which can be protonated in a medium, the pH of which is less than that of plasma or serum carry at least one free NH_3^+ function.

3. Complex according to claim 1 or 2, wherein molecules constituting a recognition signal recognized by a cell membrane receptor are present:

   . either by substitution of some of the free NH_3^+ functions of the abovementioned monomer units,
   . or on some of the abovementioned residues causing a destabilization of cell membranes,
   . or by substitution of the free NH_3^+ function of the residues causing a destabilization of cell membranes.

4. Complex according to one of claims 1 to 3, **characterized in that** the free NH_3^+ functions of the monomer units

are substituted in a ratio of about 15% to about 45%.

5. Complex according to one of claims 1 to 4, wherein the residues which can be protonated in a medium, the pH of which is less than that of plasma or serum are bases, the pK of which in an aqueous medium is less than 8, such that a proportion greater than 50% of these bases bound to a cationic polymer is not protonated in a neutral medium of pH 7.4.

6. Complex according to any one of claims 1 to 5, wherein the residues, which can be protonated in a medium, the pH of which is less than that of plasma or serum and causing destabilization of cell membranes are chosen from:

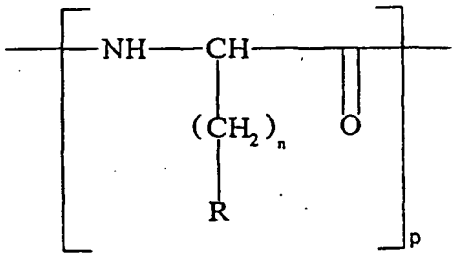
histidine, 4-carboxymethyl-imidazole, 3-(1-methyl-imidazol-4-yl)-alanine, 3-(3-methyl-imidazol-4-yl)-alanine, 2-carboxy-imidazole, histamine, 3-(imidazol-4-yl)-L-lactic acid, 2-(1-methyl-imidazol-4-yl)ethylamine, 2-(3-methyl-imidazol-4-yl) ethylamine, β-alanyl-histidine-(carnosine), 7-chloro-4-(amino-1 -methylbutylamino)-quinoline, N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine, 8-(4-amino-1-methylbutyl amino)-6-methoxyquinoline (primaquine), N^4-(6-methoxy-8-quinolinyl)-1,4-pentane diamine, quininic acid, quinolinecarboxylic acid, pteroic acid, nicotinic acid and quinolinic acid.

7. Complex according to any one of claims 1 to 6, **characterized in that** the NH_3^+ functions carried by monomer units are residues of lysine or omithine, and **in that**:

- the remaining free NH_3^+ functions of the abovementioned monomer units are also substituted to the extent of about 1% to about 60% by a molecule which constitutes a recognition signal recognized by a cell membrane receptor, this recognition signal having a molecular weight of less than 5,000,

with the proviso that the totality of the free NH_3^+ functions is of at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

8. Complex according to any one of claims 1 to 5, in which the polymer contains a polymeric grouping of the following formula (I):

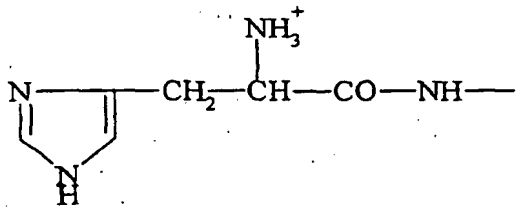


(I)

wherein:

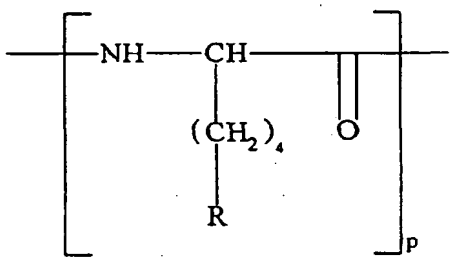
- p is an integer varying from 15 to 900,
- n is an integer varying from 1 to 6,
- this polymeric grouping contains R moieties among which:
  - 10% to 45% of the number of R moieties represent a residue carrying an imidazole nucleus
  - 10% to 90% of the number of R moieties represent free $\bar{\omega}$-amino NH_3^+.

9. Complex according to claim 8, **characterized in that** R is represented by the formula:



10. Complex according to claim 8 or 9, wherein 10% to 90% of the number of R moieties representing free $\bar{\omega}$-amino NH_3^+ are substituted to the extent of 0 to 50% by a molecule which constitutes a recognition signal.

11. Complex according to claim 8 to 10, in which the polymer comprises a polymeric grouping of the following formula (II):



in which:

- p has the meanings indicated in claim 8,
- 10% to 45% of the number of R moieties represent a residue carrying an imidazole nucleus.

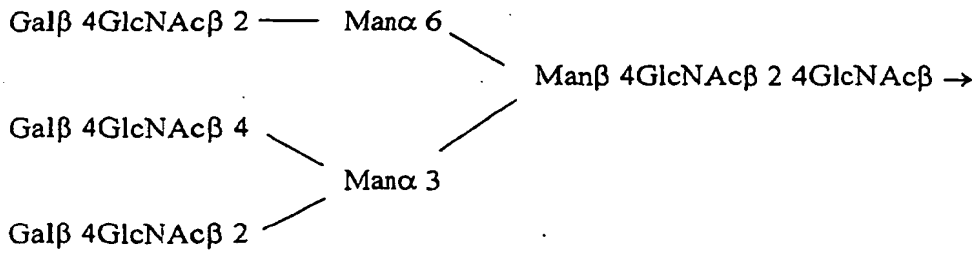
12. Complex according to claim 11, **characterized in that** the remainder of the radicals, that is to say 30% to 90% of the number of R moieties, represent $\bar{\omega}$-amino NH_3^+.

13. Complex according to claim 12, **characterized in that** 0 to 45% of the R moieties are substituted by a molecule which constitutes a recognition signal recognized by a cell membrane receptor.

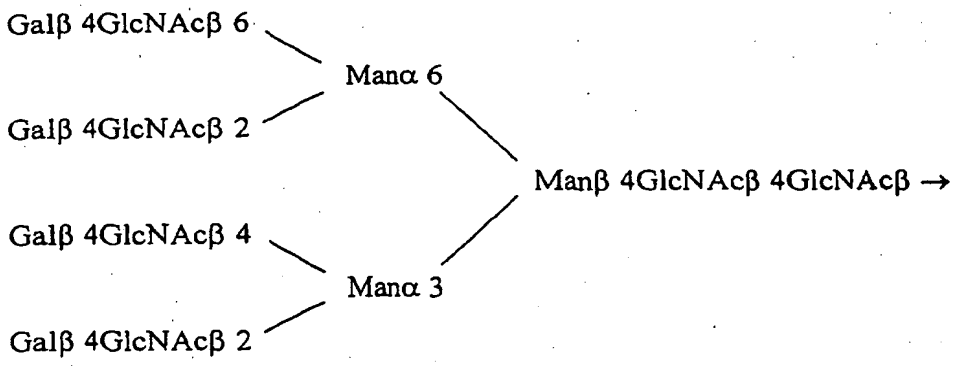
14. Complex according to one of claims 1 to 6, **characterized in that** the recognition signal is chosen from:

A) simple or complex osides recognized by membrane lectins and chosen from:

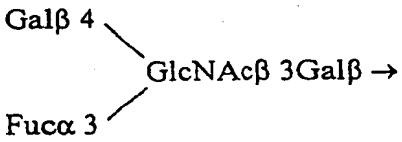
a. Asialo-oligoside of the type of triantennar lactosamine: asialoglycoprotein receptor



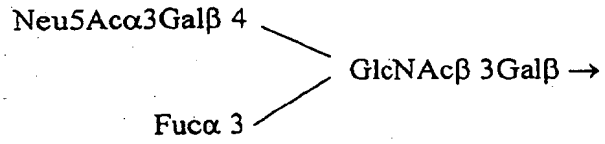
b. Asialo-oligoside of the type of tetraantennar lactosamine: asialoglycoprotein receptor



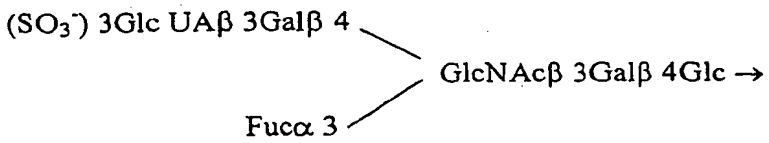
c. Lewis x:



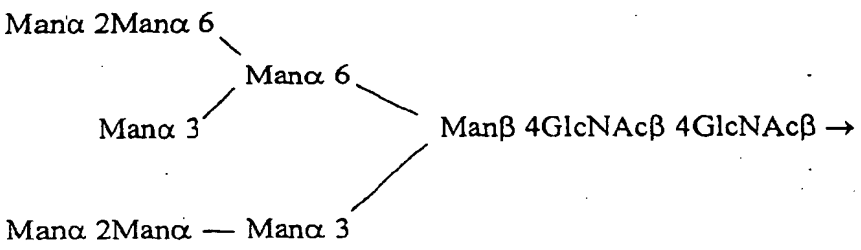
d. Lewis x sialyl: LECAM 3/2



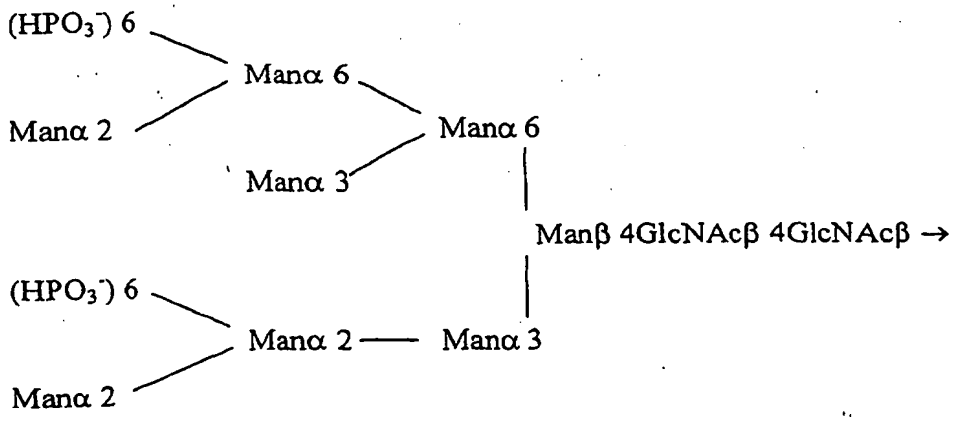
e. Sulphated Lewis x derivative (HNK1):



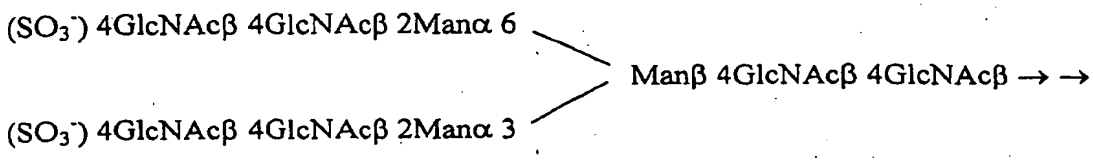
f. Oligomannoside: mannose receptor



g. Phosphorylated oligomannoside: mannose 6-phosphate receptor



h. Oligosaccharide of the type of sulphated lactosamine: sulphated GalNAc 4 receptor



B) Peptides

a) anti-inflammatory peptides or certain of their fragments recognized by receptors of the vascular wall,
b) ligand peptides of integrins,
c) chemiotactic factors,
d) peptide hormones,

C) Natural metabolites.

15. Complex according to any one of claims 1 to 7, **characterized in that** the recognition signal is chosen from:

a) marker genes,
b) genes with a therapeutic purpose,
c) genes for the purpose of vaccines.

16. Complex according to claim 15, **characterized in that** marker genes are chosen from: genes containing luciferase, green protein of the jellyfish *Aequorea victoria*, genes containing β-galactosidase, genes containing chloramphenicol acetyltransferase, and genes which confer resistance to an antibiotic.

17. Complex according to claim 15, **characterized in that** genes with a therapeutic purpose are chosen from:

- receptors of low-density lipoproteins, which are deficient in cases of hypercholesterolaemia,
- coagulation factors: factors VIII and IX,
- phenylalanine hydroxylase (phenylketonuria),
- adenosine deaminase (ADA immunodeficiency),
- lysosomal enzymes, such as β-glucosidase in the case of Gaucher's disease,
- dystrophin and minidistriphin (myopathy),
- tyrosine hydroxylase (Parkinson),

- neurone growth factors (Alzheimer),
- CFTR cystic fibrosis transmembrane conductance regulator (cystic fibrosis),
- alpha-1-antitrypsin,
- cytokines (interleukins, TNF tumour necrosing factor),
- thymidine kinase of the Herpes simplex virus,
- proteins of MHC, major histocompatibility complex, in particular HLA-B7,
- cytosine deaminase,
- genes which code for sense and antisense RNAs,
- genes which code for ribozymes.

18. Complex according to claim 15, **characterized in that** genes for the purpose of vaccines are chosen from genes which code for viral antigens (vaccination).

19. Complex according to any one of claims 1 to 10, wherein:

- the polymer is polylysine, and has a degree of polymerization of about 15 to about 900,
- the free NH_3^+ functions of the lysine units being substituted in a ratio of 35% by histidyl residues, and,
- the nucleic acid has a molecular weight of about 10^6 to about 10^8,
- the ratio between the average number of base pairs of the nucleic acid per molecule of lysine unit is about 0.2 to about 6.

20. Use of a complex according to any one of claims 1 to 19 for the *in vitro, ex vivo* or *in vivo* transfection of cells with the aid of a gene, in particular those defined in claims 17 or 18.

21. Use of a complex according to claim 19, **characterized in that** the cells are chosen from:

- cells of haematopoietic strains;
- dendritic cells;
- liver cells;
- skeletal muscle cells;
- skin cells:
  - fibroblasts,
  - keratinocytes,
  - dendritic cells,
  - melanocytes;
- cells of the vascular walls;
  - endothelial;
  - smooth muscle;
- epithelial cells of the respiratory tract;
- cells of the central nervous system;
- cancerous cells;
- cells of the immune system.

22. Method of *in vitro* or *ex vivo* transfection, **characterized in that** a complex according to any one of claims 1 to 19 is brought into contact with a medium containing cells to be transfected under conditions such that there is:

- passage of the complex from the medium into the cytoplasm of the cells,
- salting out of the nucleic acid involved in the abovementioned complex in the cytosol and/or the nucleus of the cells,
- transcription and expression of the nucleic acid in the transfected cells,
- expression of the protein corresponding to the transfected gene.

23. Pharmaceutical composition, **characterized in that** it comprises, as the active substance, at least one of the complexes according to any one of claims 1 to 19, in combination with a pharmaceutically acceptable vehicle.

24. Use of a complex according to one of claims 1 to 19, for the preparation of a medicament intended, for example, for the treatment of congenital or acquired metabolic deficiency, or the treatment of tumours, or for the preparation of a vaccine, for example a vaccine against influenza.

Histidylated Polylysine as DNA Vector: Elevation of the Imidazole Protonation and Reduced Cellular Uptake without Change in the Polyfection Efficiency of Serum Stabilized Negative Polyplexes

Mahajoub Bello Roufaï and Patrick Midoux

Centre de Biophysique Moléculaire CNRS UPR 4301 affiliated to the University of Orléans and to INSERM, 45071 Orléans Cedex 02, France

Bioconjugate Chemistry®

Reprinted from
Volume 12, Number 1, Pages 92–99

Bioconjugate Chem. **2001**, *12*, 92–99

Histidylated Polylysine as DNA Vector: Elevation of the Imidazole Protonation and Reduced Cellular Uptake without Change in the Polyfection Efficiency of Serum Stabilized Negative Polyplexes

Mahajoub Bello Roufaï and Patrick Midoux*

Centre de Biophysique Moléculaire CNRS UPR 4301 affiliated to the University of Orléans and to INSERM, 45071 Orléans Cedex 02, France. Received June 28, 2000; Revised Manuscript Received October 14, 2000

We have reported that polylysine substituted with histidyl residues (His) was suited to make complexes with plasmid DNA (pDNA) and to transfect cells in vitro in the presence of serum. The present study was performed to determine whether the acetylation of the α-amino group of histidyl residues (AcHis) had an influence on the size and the charge of polyplexes and on their transfection efficiency. We found that the presence of free α-amino groups allowed the formation of smaller polyplexes but did not modify the ζ potential of +17 mV. At a physiological salt concentration, the adsorption of many serum proteins on His- and AcHis-polyplexes reduced their size below 100 nm, inhibited their aggregation, and reversed their ζ potential to −25 mV. The acetylation of the α-amino groups reduced slightly the adsorption of serum proteins. The presence of the α-amino groups increased the p*K* of the imidazole protonation of histidine bound to polylysine from pH 5.8 to 6.9; in addition, the protonation was further elevated in the presence of pDNA. Serum stabilized negative histidylated polyplexes were less taken up by cells but their transfection efficiency did not decrease; depending on the cell line, His-polyplexes were more efficient than AcHis-polyplexes. The results indicate that (i) the α-amino groups of histidyl residues bound to polylysine favorably influence the size and the transfection efficiency of polyplexes, (ii) the α-amino groups also elevate the imidazole protonation of His-polyplexes, which is suited to destabilize the membrane of early endocytic vesicles in order to favor pDNA delivery in the cytosol, and (iii) the absorption of selective serum proteins on His-polyplexes could be a way for in vivo gene targeting.

INTRODUCTION

Soluble interpolyelectrolyte complexes (termed polyplexes) (*1*) formed between a plasmid DNA (pDNA) and a cationic polymer are a promising approach for a nonviral gene delivery (for reviews, see refs *2* and *3*). Their self-assembly in an aqueous solution results from a cooperative system between the polyanionic phosphate backbone of nucleic acids and the positive charge of amino groups of the cationic polymers via electrostatic bonds. Such electrostatic interactions allow pDNA condensation in morphologically variable polyplexes depending on the polymer structure (*4*). To be efficient, polyplexes must have the capacity (i) to enter into cells of interest, (ii) to transfer a great number of copies into the cytosol in a sufficient number of cells, and (iii) to transport a great number of copies into the cell nucleus. Polylysine and ligand-substituted polylysine are widely used to condense pDNA in toroid particles and short rods that are taken up by cells via nonspecific and receptor-mediated endocytosis, respectively. Polyplexes must escape the endocytic pathway and pDNA reach the cytosol. This is favored by using chloroquine (*5*–*7*) glycerol (*8*) or acidic dependent fusogenic peptides (*6*, *9*–*14*).

Recently, we have reported that partially histidylated polylysine complexed with pDNA actively transfect cells in culture in the presence of serum and in the absence of chloroquine or a fusogenic peptide (*15*). We found that the transfection efficiency into cystic fibrosis airway epithelial cell lines was as great as that obtained with polyethylenimine or lipofectamine as pDNA vectors (*16*). It was shown with polylysine and polyethylenimine that the polymer/DNA ratio, the chemical substitution of the cationic polymer and conditions of the polyplexes formation largely influenced the morphology, the size, the charge as well as the transfection efficiency of polyplexes (*4*, *17*–*22*).

This paper was focused on a comparative study of polyplexes made with polylysine substituted with either histidyl or *N*-acetyl-histidyl residues in order to evaluate the influence of the α-amino substitution on the transfection efficiency. The size and the charge of both polyplexes were determined as a function of the DNA/polymer ratio, the salt concentration, the pH medium and the amount of serum. Histidylated polylysines which were designed to favor pDNA delivery into the cytosol after the uptake of polyplexes by the cells, contain several imidazoles which can induce membrane destabilization after their protonation in an acidic medium. We have determined the pH of the protonation of imidazole in both polymers as well as in polyplexes and showed that the protonation of His-polyplexes[1] can occur in the lumen of early endocytic vesicles.

MATERIALS AND METHODS

Chemicals. Chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Poly(L-lysine) and HBr (degree of polymerization = 190, Bachem, Bubendorf, Switzerland) were changed in polylysine *p*-toluenesulfonate salt as previously described (*23*).

* To whom correspondence should be addressed. Phone: +33 2 38 25 55 95. E-mail: midoux@cnrs-orleans.fr.

10.1021/bc0000738 CCC: $20.00
Published on Web 12/21/2000

Histidylated Polylysine (His_{109}-pLK). Polylysine *p*-toluenesulfonate salt (50 mg; 0.87 pmol) in 3 mL of DMSO was reacted for 15 h at 20°C in the presence of diisopropylethylamine (DIEA) (23 μL; 143.5 μmol) with the *N*-succinimidyl derivative of BocHis(Boc)-OH (25.5 mg; 56.5 μmol) (Bachem). The N-protecting Boc groups of histidyl residues were removed by acidic treatment by adding H_2O/trifluoroacetic acid mixture (1:1; v:v) for 12 h at 20 °C. Water and trifluoroacetic acid were removed under reduced pressure. Histidylated polylysine was precipitated by adding 10 vol of 2-propanol and collected by centrifugation (1800*g* for 15 min). The pellet was washed with 2-propanol, collected by centrifugation, solubilized in distilled water, and freeze-dried.

***N*-Acetyl-histidylated Polylysine ($AcHis_{100}$-pLK).** Polylysine *p*-toluenesulfonate salt (50 mg; 0.87 μmol) in 3 mL of DMSO was reacted in the presence of DIEA (60 μL; 344 μmol) for 24 h at 20 °C with *N*-acetyl-His-OH (18.8 mg; 0.96 μmol) in the presence of 84.5 mg of BOP (1.92 μmol). The polymer was precipitated by adding 10 vol of 2-propanol and collected by centrifugation (1800*g* for 15 min). The pellet was washed with 2-propanol, collected by centrifugation, solubilized in distilled water, and freeze-dried.

Determination of the Histidyl Polylysine Substitution Level. The average number of histidyl residues (x) bound per pLK molecule was determined by ^{1}H NMR spectroscopy at 300 MHz in D_2O according to $x = 6(h_{8.7}/h_{Lys})$.DP, where $h_{8.7}$ was the value of the integration of the signal at 8.7 ppm corresponding to the proton (C2−H) of histidyl as well as of *N*-acetylhistidyl residues and h_{Lys} was the value of the integration, in the range 1.3−1.9 ppm, corresponding to the six methylene protons (β,γ,δ) of lysyl residues and DP was the degree of polymerization of pLK.

Fluoresceinylated Polymers. His_{109}-pLK and $AcHis_{100}$-pLK (5 mg in 0.5 mL of DMSO) were reacted for 6 h at 20°C with 5 equiv of FITC (Molecular Probes, Eugene, OR) in the presence of DIEA (2.3 μL; 14.3 μmol). Fluoresceinylated polymers were purified by precipitation in 10 vol of 2-propanol and spun down by centrifugation (800*g*, 10 min). Pellets were washed three times with 5 vol of 2-propanol in order to remove unreacted FITC. The absence of FITC was checked by silica thin-layer chromatography with a chloroform/methanol/water (6:6:1; v:v:v) mixture as solvent.

Plasmids. pSV2Luc (5.0 kb) plasmid was an expression vector encoding the firefly luciferase gene under the control of the SV40 T large antigen promoter (*24*). pUT650 plasmid (5.15 kb) (CAYLA, Toulouse France) was an expression vector encoding the firefly gene under the control of the human cytomegalovirus promoter.

Polyplexes Formation. A polymer in a total volume of 30 μL of distilled water containing 5% (w/v) glucose unless otherwise stated was mixed with 10 μg of pDNA in 70 μL of distilled water containing 5% (w/v) glucose. The mixture was kept 30 min at 20 °C to allow the formation of polyplexes.

ζ Potential Measurement. The global surface charge of polyplexes was measured by electrophoretic mobility using a ZetaSizer 3000 (Malvern Instruments, Orsay, France) with the following parameters: viscosity, 0.89 cP; dielectric constant, 79; temperature, 25 °C; Smoluchowsky constant F(ka), 1.50; maximum voltage of the current, 15 V. The system was routinely calibrated using a −50 ± 5 mV standard dts 5050 from Malvern. The measurement was performed five times for 30 s with zero field correction in aqueous dep cell on 1.1 mL samples containing a final DNA concentration of 10 μg/mL.

Size Measurement. Polyplexes size was determined by quasi-elastic laser light scattering (QELS) using a ZetaSizer 3000 with a sample refractive index of 1.59; viscosity, 0.89 cP. The system was routinely calibrated using a 220 nm standard dts 5050 from Malvern. For serum free polyplexes, measurements were performed 10 times for 12 s on 1 mL of sample. The size corresponds to an average size calculated by using the cumulant analysis mode.

Imidazole Protonation of Histidylated Polymers. The protonation of the imidazole of histidine free base (5 mg/mL) and of histidylated polymers (10 mg/mL) was assessed by measuring the chemical shift of the C2−H of histidyl residues versus the pH medium by using ^{1}H NMR spectroscopy in D_2O at 300 MHz. Chemical shifts were determined downfield the HDO peak at 4.75 ppm used as the internal standard.

Effect of Serum on the ζ Potential of Polyplexes. Polyplexes prepared as described above were diluted in 1 mL of 5 mM Hepes buffer, pH 7.4, containing 10 or 20% of 0.2 μm filtered fetal bovine serum (FBS, Gibco, Renfrewshire, U.K.). After 30 min, the solution was loaded on 50% (w/v) simple sucrose cushion and centrifuged at 4 °C for 4 h at 30 000 rpm in a Beckman Ultracentrifuge SW40Ti rotor. The supernatant was carefully removed and the pellet containing polyplexes was resuspended in 200 μL of distilled water. An agarose gel electrophoresis of the suspension showed that more than 90% of polyplexes was in the pellet. ζ-Potential measurement was performed as described above after dilution in 1 mL of distilled water.

Cells and Cell Culture. Human hepatocarcinoma cells (HepG2 cells, ATCC HB 8056, Rockville, MA) were cultured in Eagle's minimal essential medium (MEM, Gibco) with 10% heat inactivated FBS. The immortalized human tracheal epithelial cell line (ΣCFTE29o- cells) was from a cystic fibrosis patient homozygous for the ΔF508 mutation and was kindly given by D. C. Gruenert (Cardio-vascular Researched Institute, San Fransisco, CA). ΣCFTE29o- cells were plated on tissue culture plasticware coated with fibronectin, collagen, and bovine serum albumin and grown in MEM supplemented with 10% non decomplemented FBS (*25*). All culture media were supplemented with 2 mM L-glutamax (Gibco) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin from Gibco). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Cells were harvested by treatment with PET (PBS with 0.02% (w/v) EDTA and 2.5 μg/mL trypsin) at 37 °C for 5 min. Cells were mycoplasma free as evidence by bisbenzimidazole (Hoechst 33258) (*26*).

Uptake of Histidylated Polylplexes. HepG2 and ΣCFTE29o- cells (4×10^5 cells) were incubated for 3 h at 37 °C in the presence of fluorescein-labeled polyplexes at 10 μg/mL pDNA made with either *F*-His_{109}-pLK (30 μg) or *F*-$AcHis_{109}$-pLK (40 μg) and chased 1 h in the presence of the respective unlabeled polyplexes at the same concentration. Cells were washed three time with PBS, trypsinized for 5 min at 37 °C and suspended in cold PBS. The cell-associated fluorescence was analyzed

[1] Abbreviations: AcHis, *N*-acetyl-histidyl residue; BOP, benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate; EDTA, ethylenediaminetetracetic acid; FITC, fluorescein isothiocyanate isomer I; *F*−, fluorescein-labeled; His, histidyl residue; MEBS, 1 mM 3-[*N*-morpholino] propane sulfonic acid, 0.1 mM EDTA, 10 mM NaCl; PBS, phosphate-buffered saline; pLK, poly-L-lysine; QELS, quasi-elastic laser light scattering; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

using a FACSort flow cytometer (Becton Dickinson, Grenoble, France) before and after a postincubation for 15 min at 4 °C in the presence of 50 μM monensin (*27, 28*). Data were analyzed using Cell Quest Software (Becton Dickinson).

Polyfection and Luciferase Assay. Two days prior the transfection, cells were seeded at 2×10^5 cells per 4 cm^2 in a 12 wells plate and incubated at 37 °C in a humidified atmosphere (5% CO_2). The polyplexes were prepared as described above in 100 μL of water containing 5% (w/v) glucose and then diluted with 900 μL of culture medium containing either 0, 10, or 20% FBS. Cells were then incubated at 37 °C in 5% CO_2 with polyplexes. After 4 h, the medium containing polyplexes was removed and replaced by culture medium containing 10% FBS without polyplexes. Luciferase gene expression was measured at 72 h by monitoring the luminescence activity according to De Wet et al. (*29*). The medium was discarded, and cells were washed three times with PBS. The homogenization buffer (200 μL of 8 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1% Triton ×100, 15% glycerol, 25 mM Tris-phosphate buffer, pH 7.8) was poured into each well. After 15 min at 20 °C, the solution was recovered and spun down (5 min at 800 g). Ninety five μL of a 2 mM ATP solution in the homogenization buffer without Triton x 100 was added to 60 μL of supernatant and the solution was shaken with a vortex. The luminescence was recorded for 4 s in a Lumat LB 9501 luminometer (Berthold, Wildbach, Germany) upon addition of 150 μL of a 167 mM luciferin solution in water. Measurements were done in duplicate. The luciferase activity was expressed as the number of relative light units (RLU) per cell. The number of RLU of 1 pg/mL of luciferase was 2000 under these conditions.

RESULTS

Polyplexes Formation. The condensation of pDNA in the presence of histidylated polylysines at low salt concentration was assessed by agarose gel electrophoresis, ζ potential and size measurements of polyplexes. The migration in the agarose gel showed that 10 μg of pDNA mixed with 30 μg of His_{109}-pLK or 30 μg of $AcHis_{100}$-pLK was completely retarded and thus all the plasmid was complexed whereas pDNA was completely retarded with 10 μg of pLK (Figure 1). At pH 7.4, polyplexes made with 30 μg of His_{109}-pLK and 10 μg of pDNA at a N:P of 3.4 (ratio of lysyl ϵ-amino plus histidyl α-amino groups to pDNA phosphates) were positively charged and exhibited a ζ potential of +18 ± 2 mV (Table 1). Increasing the amount of polymer did not increase the ζ potential. Polyplexes made with 30 μg of polylysine at the same N:P of 3.4 (ratio of lysyl ϵ-amino groups to pDNA phosphates) exhibited a similar ζ potential (+17 mV) which increased to + 22 ± 3 mV with 40 μg of pLK. Polyplexes made with 40 μg of $AcHis_{100}$-pLK (N:P, lysyl ϵ-amino groups to DNA phosphates ratio of 2.3) exhibited a ζ potential of +14 ± 3 mV close to that of polyplexes made with His_{109}-pLK at a N:P of 3.4 (Table 1). QELS measurements showed that polyplexes made with His_{109}-pLK gave homogeneous particles of mean diameter 110 ± 15 nm under maximal condensation which were slightly bigger than those made with pLK (Table 1). $AcHis_{100}$-pLK formed larger polyplexes (165 ± 15 nm) than His_{109}-pLK indicating that with the latter the α-amino groups interacted with pDNA phosphates and induced a better condensation. These results indicated that 10 μg of pDNA was completely condensed and saturated with 30 μg of His_{109}-pLK. In water, the presence of histidyl as well as *N*-acetyl-histidyl residues on

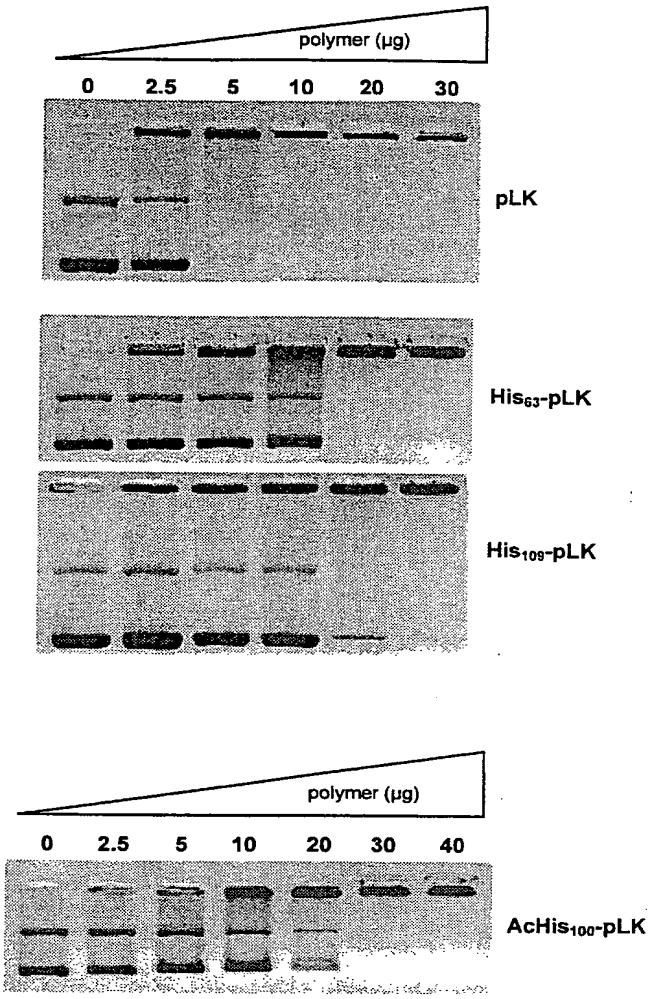


Figure 1. Agarose gel electrophoresis of polyplexes. Various amounts of cationic polymer expressed in micrograms per milliliter (μg/mL) were mixed with 10 μg of plasmid in 60 μL of distilled water. After 30 min incubation at 20 °C, 20 μL of each sample was analyzed by electrophoresis for 2 h under 80 V/cm, through a 0.6% agarose gel containing ethidium bromide (1 μg/mL of gel) in Tris-borate-EDTA buffer (95 mM Tris, 89 mM boric acid, and 2.5 mM EDTA), pH 8.6.

polylysine increased 2-fold the global charge of polyplexes (+33 mV). The enhancement of the ζ potential due to the presence of protonated imidazoles shows that protonated imidazoles do not interact with pDNA phosphates probably because of their distance between the polymer backbone.

Effect of the Salt Concentration on the Polyplexes Size. We have investigated the influence of the salt concentration on the size of histidylated polyplexes because it is known that at physiological salt concentration (i.e., 0.15 M NaCl) pLK/DNA complexes form aggregates. QELS measurements showed that in distilled water and water containing 5% glucose, the size of polyplexes was 120 ± 10 and 105 ± 15 nm, respectively (Figure 2). Small polyplexes of 100 nm were also obtained after dilution in MEBS, pH 7.2, or 5 mM Hepes, pH 7.0. However, aggregates appeared as soon as the salt concentration was 25 mM NaCl and large aggregates of 800 nm were formed at 0.15 M NaCl. Large aggregates were also observed when the polyplexes were prepared at physiological salt concentration in 0.15 M NaCl serum-free medium.

Table 1. Size and ζ Potential of Histidylated Polyplexes[a]

	polymer/pDNA weight ratio (μg/μg)					
	ζ potential (mV)			size (nm)		
	2	3	4	2	3	4
His_{109}-pLK	+17.5 ± 3 (+32)*	+18 ± 2 (+33)	+17 ± 3 (+34)	140 ± 10	110 ± 10	110 ± 10
$AcHis_{100}$-pLK	−18 ± 2 (+32)	−5 ± 3 (+34)	+14 ± 3 (+37)	179 ± 10	170 ± 10	165 ± 15
pLK	+10 ± 3 (+12)	+17 ± 3 (+18)	+22 ± 3 (+22)	100 ± 10	100 ± 10	90 ± 10

[a] Polyplexes were prepared by mixing 10 μg of pDNA with different amounts of cationic polymers in 100 μL of water containing 5% (w/v) glucose. The mixture was kept for 30 min at 20 °C and diluted with 900 μL of either 5 mM Hepes buffer, pH 7.4, or (*) water containing 5% glucose. Size and ζ potential measurements were performed by using the ZetaSizer 3000.

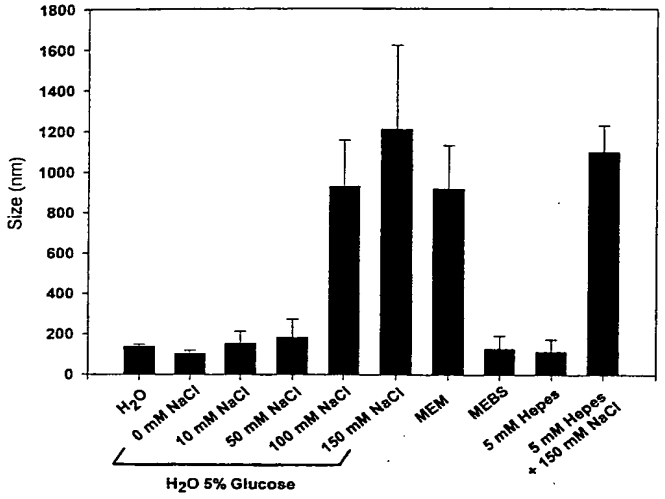


Figure 2. Effect of the salt concentration on the polyplexes size. pDNA/His-pLK polyplexes (10/30; μg/μg) were prepared in 100 μL of distilled water. After 30 min at 20 °C, they were diluted with 900 μL of either distilled water, water containing 5% glucose and various NaCl concentration, serum-free MEM or other buffers. Polyplexes size was then determined after 1 h by QELS measurements with the ZetaSizer 3000.

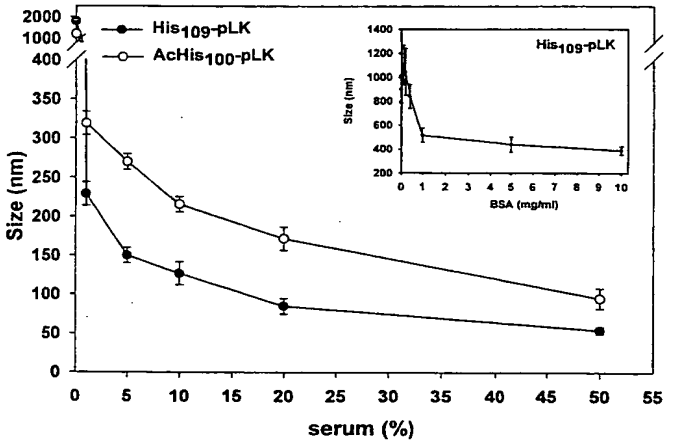


Figure 3. Influence of fetal bovine serum (FBS) on the polyplexes size. pDNA/His_{109}-pLK (10/30; μg/μg) and pDNA/$AcHis_{100}$-pLK (10/40 μg/μg) polyplexes were prepared in 100 μL of H_2O 5% glucose. After 30 min at 20 °C, they were diluted with 900 μL of 0.15 M NaCl 5 mM Hepes buffer, pH 7.4 containing various amounts of FBS and the solution was kept 1 h at 20 °C. QELS measurements were performed upon 10 times dilution of polyplexes at a final serum concentration less than 2.5% (v/v) in order to prevent any significant light scattering signal coming from serum components. (Inset) *Influence of bovine serum albumin (BSA) on the polyplexes size.* pDNA/His_{109}pLK polyplexes were diluted 10 times in 0.15 M NaCl 5 mM Hepes buffer, pH 7.5, containing various amount of BSA.

Effect of Serum on the Size and the Charge of Polyplexes. As shown above, the size of polyplexes grown up rapidly in the absence of serum and formed aggregates of 1−3 μm within 30 min (Figure 3). In contrast, the size of polyplexes was stabilized at 250 ± 10 and at 100 ± 10 nm when polyplexes prepared at low salt concentration were diluted 10 times in 0.15 M NaCl buffer containing 1 and 10% serum, respectively (Figure

The hydrodynamic diameter of polyplexes was further reduced to 70 ± 5 and 50 ± 5 nm in the presence of 20 and 50% serum, respectively. This inhibition of the aggregation and the reduction of the size of polyplexes in the presence of serum was also observed with AcHis-polyplexes but their size reached only 90 ± 10 nm in the presence of 50% serum (Figure 3). These data indicate that serum anionic components bind to the surface of cationic polyplexes and prevent hydrophobic contacts between the particles and/or modify the global surface charge allowing repulsion between particles. First of all, we found that the size of polyplexes decreased and reached a minimum of 375 nm in the presence of 10 mg/mL serum albumin a protein present in large quantity in the serum (Figure 3, inset). The size was bigger than that obtained in the presence of serum suggesting that other proteins interacted with polyplexes to form particles of 70 nm. Then, we have performed SDS−PAGE analysis of polyplexes after incubation in the presence of bovine fetal serum and isolation by ultracentrifugation on sucrose cushion. We found that several major serum proteins of 66 (probably serum albumin), 59, 52, 45, and 27 kDa molecular mass (Figure 4, lane j) were associated with both histidylated polyplexes (Figure 4, lanes d−g). In addition, there were several other proteins with high, medium (between 30 and 45 kDa) and low (10, 14, and 16 kDa) molecular mass present in low quantity in the serum that were more strongly associated with His-polyplexes (Figure 4, lanes d and e). The acetylation of the α-amino group of histidyl residues reduced drastically the binding of all serum proteins (Figure 4, lanes f and g).

The global surface charge of polyplexes incubated with 10 and 20% serum was also investigated after their isolation by ultracentrifugation on sucrose cushion. His- and AcHis-polyplexes exhibited a negative ζ potential of −25 and − 15 mV, respectively (Figure 5).

Influence of pH Medium on Polyplexes Size. The size of His-polyplexes increased from 230 nm at pH 7.5 to 350−430 nm at pH 9.5 due to a decrease of the interactions between His-pLK and pDNA after deprotonation of α-amino groups (p*K* close to pH 7.0) when the pH of the medium was above 7.5 giving less condensed polyplexes (Figure 6). The size decreased to 100 nm at

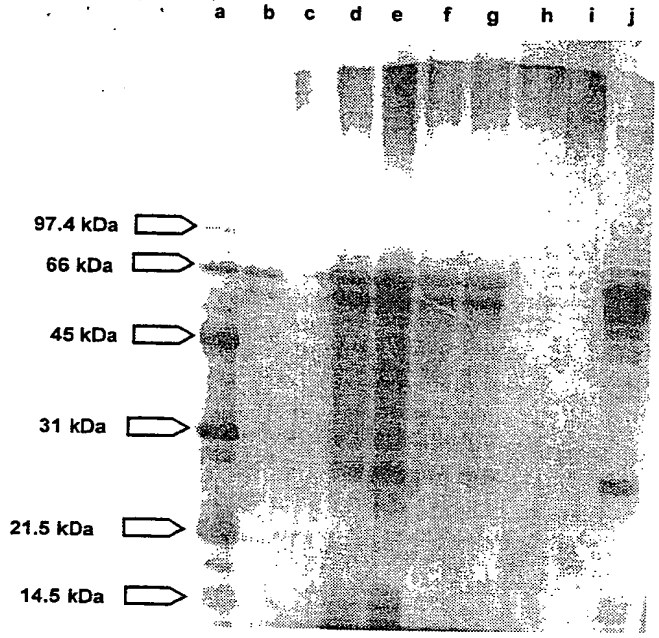


Figure 4. SDS–PAGE analysis of serum proteins bound to His- and AcHis-polyplexes. Polyplexes containing 10 μg/mL pDNA in 100 μL of H_2O 5% glucose were diluted in 5 mM Hepes buffer, pH 7.4, containing filtered bovine fetal serum. After 30 min, polyplexes were isolated by ultracentrifugation on 50% (w/v) sucrose cushion and analyzed under SDS–PAGE with visualization by a silver staining. (Lane a) Molecular weight markers; (lane b) 100 times diluted filtered serum; (lane c) His-polyplexes without serum; (lane d) His-polyplexes with 10% serum; (lane e) His-polyplexes with 20% serum; (lane f) AcHis-polyplexes with 10% serum; (lane g) AcHis-polyplexes with 20% serum; (lane h) histidylated polylysine; (lane i) plasmid DNA; (lane j) serum without polyplexes.

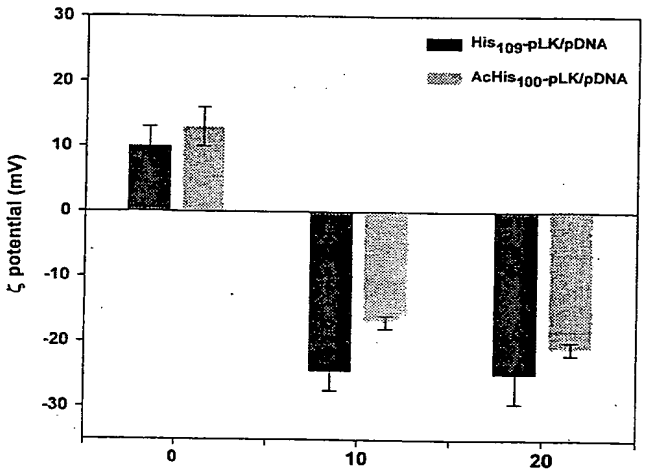


Figure 5. Influence of serum on polyplexes charge. (Dark bar) pDNA/His_{109}-pLK and (shaded bar) pDNA/$AcHis_{109}$-pLK complexes were prepared in 100 μL of H_2O. After 30 min at 20 °C, polyplexes were diluted in 900 μL of 5 mM Hepes buffer, pH 7.4, in the absence or the presence of bovine fetal serum and the solution kept 30 min at 20 °C. Polyplexes were then isolated by ultracentrifugation on 50% (w/v) sucrose cushion. ζ potential measurements were performed on resuspended polyplexes.

pH 6.5 and remained stable at lower pH when the α-amino groups were protonated. In contrast, the size of AcHis-polyplexes (165 nm) did not depend on the pH of the medium.

Imidazole Protonation. The protonation of the imidazole in His_{109}-pLK, $AcHis_{100}$-pLK and histidine free base

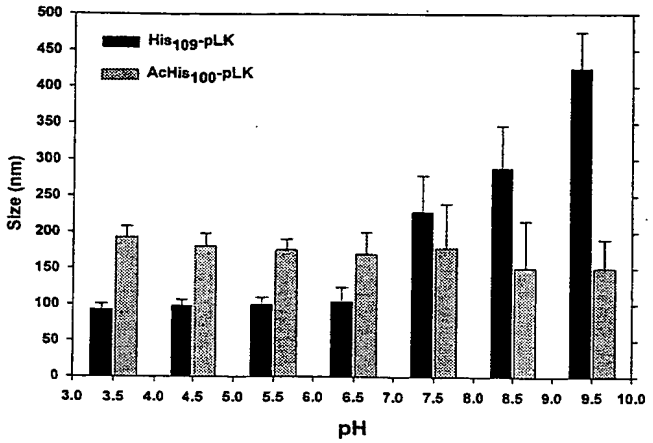


Figure 6. Influence of pH medium on the polyplexes size. pDNA (10 μg) was complexed with either (dark barr) His_{109}-pLK (30 μg) or (shaded barr) $AcHis_{100}$-pLK (40 μg) for 30 min in 100 μL of 5 mM Hepes buffer, pH 7.4, and diluted 10 times in 5 mM Hepes buffer, pH 7.4. The pH medium was then adjusted under pH meter by adding aliquots of either a 0.1 M HCl or a 0.1 M NaOH solution and the size of polyplexes was measured.

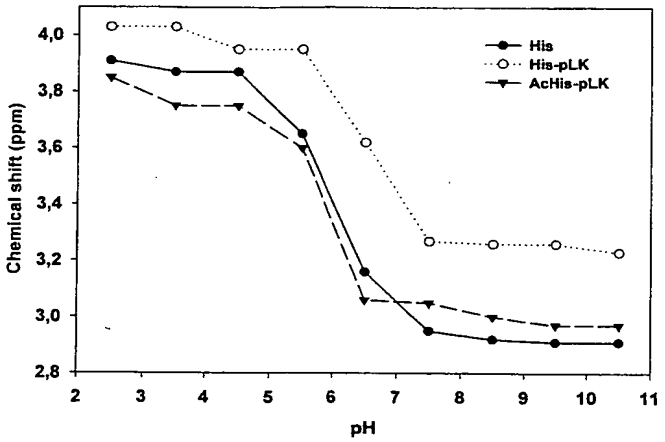


Figure 7. Imidazole protonation in histidylated polylysines. The chemical shifts of imidazole C2-H of either (○) His-pLK, (▼) AcHis-pLK, or (●) histidine reflecting the charge state of the imidazole ring was measured by ^1H NMR spectroscopy in D_2O at 300 MHz as a function of the pH medium. Chemical shifts were determined downfield from the HDO peak at 4.75 ppm used as the internal standard.

was determined by ^1H NMR spectroscopy by monitoring the C2–H peak position versus the pH medium reflecting the charge state of the imidazole ring (*30*). The p*K* of the imidazole in histidine free base, His_{109}-pLK, and $AcHis_{100}$-pLK was found to be 5.8, 6.9, and 6.0, respectively (Figure 7). The protonation of the imidazole of a *N*-acetyl-histidyl residue bound to polylysine had a p*K* similar to that of histidine free base. In contrast, the protonation occurred at a 1.1 pH unit higher than in histidine free base when the α-amino groups were free.

The imidazole protonation was also investigated in polyplexes by measuring their ζ potential at various pH. Measurements were performed with polyplexes made with both histidylated polylysines but only data from ζ potential values of those made with $AcHis_{100}$-pLK were used to evidence the protonation of the imidazole. The ζ potential values of polyplexes made with His_{109}-pLK did not only correspond to the imidazole protonation but also to that of the α-amino group which occurs between pH 7.0 and 6.5. The ζ potential of AcHis-polyplexes increased

Table 2. Uptake of Histidylated Polyplexes and Luciferase Activity into ΣCFTE29o- and HepG2 Cells

		ΣCFTE29o-			HepG2		
		cell fluorescence[a]		luciferase	cell fluorescence[a]		luciferase
polymer	FBS (%)	−Mo	+Mo	activity[b]	−Mo	+Mo	activity[b]
His_{109}-pLK	0	902	1572	46	243	638	22
	10	363	643	109	140	248	28
	20	286	414	93	132	233	23
$AcHis_{100}$-pLK	0	1386	3867	35	565	2845	17
	10	946	1642	28	250	1642	23
	20	952	1478	24	216	1690	35

[a] Mean of the cell-associated fluorescence (arbitrary units) measured by flow cytometry before (−Mo) and after (+Mo) a posttreatment with monensin. The cell-associated fluorescence was expressed relative to that of cells incubated with His_{109}−pLK/pDNA polyplexes. [b] Luciferase activity was expressed as the relative light unit (RLU) per cell.

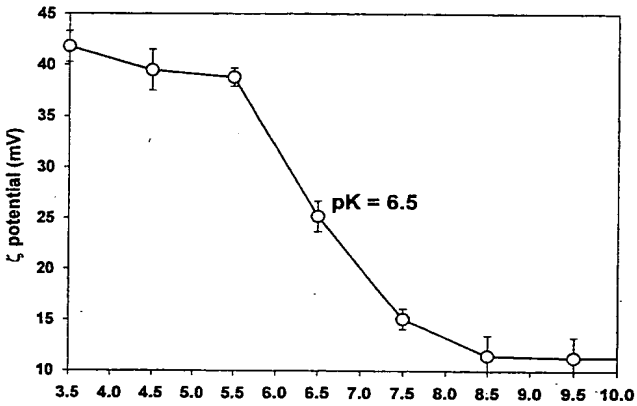


Figure 8. Imidazole protonation in *N*-acetyl-histidylated polyplexes. pDNA (10 μg) was complexed with $AcHis_{109}$-pLK (40 μg) in 200 μL of 5 mM Hepes buffer, pH 7.4, the solution kept 30 min at 20 °C and then diluted 10 times in 5 mM Hepes buffer pH 7.4. The pH was adjusted under pH meter by adding aliquots of either a 0.1 M HCl or 0.1 M NaOH solution and the ζ potential of polyplexes was measured.

from +5 to +40 mV when the pH decreased from pH 7.5 to 5.0 as a consequence of the protonation of the imidazole (Figure 8). In polyplexes, the p*K* of the imidazole was estimated to 6.5 which was slightly higher than that found for the polymer in the absence of plasmid (p*K* = 6.0) (Figure 7).

Uptake of Histidylated Polyplexes. HepG2 and ΣCFTE29o- cells were incubated with pDNA complexed with either *F*-His-pLK or *F*-AcHis-pLK. After 3 h, the medium was removed and cells were chased 1 h in the presence of unlabeled polyplexes. The cell-associated fluorescence was then analyzed by flow cytometry after postincubation with the proton/sodium ionophore monensin to evidence the uptake of polyplexes. Monensin induces neutralization of acidic compartments and restores the fluorescence of fluorescein quenched in an acidic medium such as in endosomes and lysosomes (27, *28*). The fluorescence intensity of both cell lines incubated in the absence of serum with His-polyplexes and AcHis-polyplexes was 2−3-fold higher in average than that of cells incubated in the presence of serum (Table 2). This showed that polyplexes aggregates generated in the absence of serum were more associated with the cells than the 100 nm stabilized polyplexes in the presence of serum. Indeed, the number of polyplexes molecules was increased on the surface of adherent cells because the aggregates sedimented rapidly in serum-free medium; in addition, their positive ζ potential favored their binding to sulfated proteoglycans of the plasma membrane. Conversely, the binding and the uptake by the cells of small polyplexes which exhibited a negative ζ potential were reduced in the presence of serum (Table 2). AcHis-polyplexes were more associated with both cell lines than His-polyplexes probably because their size was bigger and their sedimentation faster than His-polyplexes. After a posttreatment with monensin, the cell-associated fluorescence of ΣCFTE29o- cells incubated with both polyplexes increased 2-fold showing that some polyplexes were internalized in acidic compartments (Table 2). The cell-associated fluorescence of HepG2 cells incubated with *F*-AcHis-polyplexes was 5−7.8-fold higher after monensin posttreatment. This indicated that these polyplexes were internalized in more acidic compartments than in ΣCFTE29o- cells or that a greater amount of polyplexes were internalized in the same compartments as in ΣCFTE29o- cells.

Polyfection with Histidylated Polyplexes. The luciferase activity into the cell lines transfected with both histidylated polyplexes was similar in the absence of serum, but the luciferase activity was 2-fold greater in ΣCFTE29o- cells (Table 2). The luciferase activity into ΣCFTE29o- cells transfected with His-polyplexes in the presence of serum was 2-fold greater than that obtained in the absence of serum; it was also 4−5-fold greater than into cells transfected with AcHis-polyplexes (Table 2). This indicates that small polyplexes are more efficient in transfecting cells and that pDNA condensation is likely to play a role in the uptake and the intracellular processing of polyplexes which could depend on the cell type. Indeed, the luciferase activity into HepG2 cells transfected with both histidylated polyplexes was similar and did not depend on the presence of serum (Table 2).

DISCUSSION

The assembly of pDNA with histidylated polylysine at low ionic strength via both lysyl ϵ-amino and histidyl α-amino groups, gave homogeneous small particles with a positive ζ potential. As reported for polylysine and polyethylenimine (*17*−*22*), histidylated polyplexes showed high tendency to aggregate in 0.15 M NaCl and serum-free medium. In contrast, the polyplexes particles remained stable and kept a small size in 10% serum supplemented medium. This is due to the adsorption of serum proteins on the polyplexes surface which then prevents hydrophobic particle/particle interactions. Beside the major proteins of the serum including serum albumin, several other proteins with low molecular masses and molecular masses ranging between 30 and 45 kDa difficult to identify were strongly associated with His-polyplexes. They might be proteins with acidic pI such as α1-fetoprotein (66 kDa), α1-acid glycoprotein (41 kDa), apolipoproteins (A-I , 30 kDa; E, 34 kDa; A-II, 8.5 kDa) and α1-microglobulin (31 kDa). The binding of these serum proteins probably occurred via interactions with protonated imidazole of His-polyplexes. Indeed, in agreement with the respective protonation of imidazole, their

binding at pH 7.4 on AcHis-polyplexes was reduced because the number of protonated imidazole in AcHis-polyplexes was lower than in His-polyplexes. While the ζ potential of both polyplexes was similar, a different distribution of the charge on the surface of polyplexes could also explain a decrease of the binding of serum proteins on AcHis-polyplexes. By using the same protocole but with plasma which instead of serum contains high molecular weight proteins of the coagulation cascade, pLK/DNA complexes were found to bind proteins with molecular masses of 66 (probably serum albumin) and 120 kDa (*31*, *32*). Polyplexes with polyethylenimine were found to bind high molecular mass plasma proteins (up to 150 kDa) such as IgM, fibronectin, fibrinogen, complement C3 and albumin, but in this case the isolation of polyplexes was carried out by filtration (*33*).

In addition to the inhibition of the polyplexes aggregation, the binding of serum proteins lead to polyplexes with a net negative ζ potential (−25 mV). This reversion of the global charge of polyplexes decreased their uptake by the cells but did not decrease their transfection efficiency; it even increased 2-fold for ΣCFTE29o- cells. This suggests that a preincubation of polyplexes in autologous serum could be a way to decrease the nonspecific cell binding via the negatively charged sulfated proteoglycans (*34*) and might be suited to overcome the activation of the alternative complement pathway by cationic particles (*35*). Identification of serum proteins that bind strongly to His-polyplexes could be useful for in vivo gene targeting.

Histidylated polylysines contain several protonable imidazoles which are supposed to induce membrane destabilization after protonation in an acidic medium. The protonation of the imidazoles appears more sensitive to the presence of the α-amino group of the histidyl residues than that of the ϵ-amino group of the lysyl residues on the polymer. The protonation of 50% of the imidazoles of His-pLK occurs at pH 6.9 whereas that of AcHis-pLK occurs at pH 6.0. The imidazole protonation is also modified when the polymer is complexed with pDNA; in AcHis-polyplexes it occurs at pH 6.5, at a 0.5 pH unit higher than in AcHis-pLK. This elevation of the p*K* is consistent with that reported for polyhistidine/DNA complexes (*36*) and for Poly(Lys,His) copolymer/DNA complexes (*37*). In the latter protonated imidazoles were found to interact with DNA phosphates. Therefore, the imidazole protonation of histidylated polyplexes can surely occur in endocytic vesicles following acidification their lumen from pH 7.4 to 6.0 and can allow the destabilization of vesicles to deliver pDNA into the cytosol. However, the mechanism leading to pDNA delivery into the cytosol in the presence of His-pLK is not yet known. Membrane could be disrupted via a direct interactions of His-pLK as for H5WYG peptide (*14*). This peptide (GLFHAIAHFIHGGWHGLIHGWYG) containing five histidines permeabilizes the plasma membrane of cells at pH 6.1 with a midpoint at pH 6.8. The involvement of the imidazole protonation in the pDNA delivery in the cytosol is also supported by confocal microscopy data showing that histidylated oligolysines induce a cytosolic delivery of antisense oligonucleotides after their uptake by cells via acidic vesicles (*38*). In addition, the effect of H5WYG, histidylated oligolysines and histidylated polylysines are inhibited in the presence bafilomycin A1 an inhibitor of the ATPase proton pump of endosomes. However, the imidazole protonation of histidylated polyplexes could also buffer the lumen of vesicles containing polyplexes and induce swelling and destabilization of vesicles as for chloroquine and polyethylenimine.

In conclusion, this study shows that the presence of free α-amino group on the histidyl residues on His-pLK allows the formation of small and soluble polyplexes. It shifts the imidazole protonation up to a neutral pH which is in favor for a destabilization of very early endocytic vesicles. The adsorption of serum proteins especially on His-polyplexes which (i) renders the particles negatively charged, (ii) reduces their nonspecific binding on the cells, (iii) stabilizes particles with a small size, and (iv) does not affect their transfection efficiency, points out that selective proteins recognized by a given cell type could be used for in vivo gene targeting.

ACKNOWLEDGMENT

We thank Brigitte Guérin for her skillful assistance in cell culture and plasmid preparation; Henri Labbé and Anita Caille for they skillful assistance in NMR spectroscopy; Nadège Piclin for her technical help in size measurements. This work was partly supported by grants from Agence Nationale de Recherche sur le Sida (ANRS, no. 97003), Association de Recherche sur le Cancer (ARC), EU (Bio 4- CT97-2216) and Association Francaise de Lutte contre Ia Mucoviscidose (AFLM). P.M. is Research Director at INSERM. M.B.R. received a fellowship from AFLM.

LITERATURE CITED

(1) Felgner, P. L., Barenholz, Y., Behr, J. P., Cheng, S. H., Cullis, P., Huang, L., Jessee, J. A., Seymour, L., Szoka, F., Thierry, A. R., Wagner, E., and Wu, C. (1997) Nomenclature for synthetic gene delivery systems. *Hum. Gene Ther. 8*. 511−512.

(2) Kabanov, A. V., and Kabanov, V. A. (1995) DNA complexes with polycations for the delivery of genetic material into cells. *Bioconjugate Chem. 6*, 7−20.

(3) Mahato, R. I., Smith, L. C., and Rolland, A. (1999) Pharmaceutical perspectives of nonviral gene therapy. *Adv. Genet. 41*, 95−156.

(4) Tang, M. X., and Szoka, F. C. (1997) The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. *Gene Ther. 4*, 823−832.

(5) Zenke, M., Steinlein, R., Wagner, E., Cotten, M., Beng, H., and Birnstiel, M. L. (1990) Receptor-mediated endocytosis of transferrin-polycation conjugates an efficient way to introduce DNA into hematopoetic cells. *Proc. Natl. Acad. Sci. U.S.A. 87*, 3655−3659.

(6) Midoux, P., Mendes, C., Legrand, A., Raimond, J., Mayer, R., Monsigny, M., and Roche, A. C. (1993) Specific gene transfer mediated by lactosylated poly-L-lysine into hepatoma cells. *Nucleic Acids Res. 21*, 871−878.

(7) Erbacher, P., Roche, A. C., Monsigny, M., and Midoux, P. (1996) Putative role of chloroquine in gene transfer into a human hepatoma cell line by pDNA/lactosylated polylysine complexes. *Exp. Cell Res. 225*, 186−194.

(8) Zauner, W., Kichler, A., Schmidt, W., Sinski, A., and Wagner, E. (1996) Glycerol enhancement of ligand polylysine/DNA transfection. *BioTechniques 20*, 905−913.

(9) Wagner, E., Zatloukal, K., Cotten, M., Kirlappos, H., Mechtler, K., Curiel, D. T., and Birnstiel, M. L. (1992) Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. *Proc. Natl. Acad. Sci. U.S.A. 89*, 6099−6103.

(10) Plank, C., Oberhauser, B., Mechtler, K., Koch, C, Wagner, E. (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. *J. Biol. Chem. 269*, 12918−12924.

(11) Haensler, J., and Szoka, F. C., Jr. (1993) Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjugate Chem. 4,* 372−379.
(12) Gottschalk, S., Sparrow, J. T., Hauer, J., Mims, M. P., Leland, F. E., Woo, S. L. C., and Smith, L. C. (1996) A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells. *Gene Ther. 3*, 448−457.
(13) Zauner, W., Blaas, D., Kuechler, E., and Wagner, E. (1995) Rhinovirus-mediated endosomal release of transfection complexes. *J. Virol. 69*, 1085−1092.
(14) Midoux, P., Kichler, A., Boutin, V., Maurizot, J. C., and Monsigny, M. (1998) Membrane permeabilization and efficient gene transfer by a peptide containing several histidines. *Bioconjugate Chem. 9*, 260−267.
(15) Midoux., P., and Monsigny, M. (1999). Efficient gene transfer by histidylated polylysine/pDNA complexes. *Bioconjugate Chem. 10*, 406−411.
(16) Fajac, I., Allo J.-C., Souil, E., Merten, M., Pichon, C., Figarella, C., Monsigny, M., Briand, P., and Midoux, P. (2000) Histidylated polylysine as a synthetic vector for gene transfer into immortalized cystic fibrosis airway surface and airway gland serous cells. *J. Gene Med. 2*, 368−378.
(17) Perales, J. C., Ferkol, T., Beegen, H., Ratnoff, O. D., and Hanson, R. W. (1994) Gene transfer in vivo: sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake. *Proc. Natl. Acad. Sci. U.S.A. 91*, 4086−4089.
(18) Wagner, E., Cotten, M., Foisner, R., and Birnstiel, M. L. (1991) Transferrin-polycation−DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. *Proc. Natl. Acad. Sci. U.S.A. 88*, 4255−4259.
(19) Wolfert, M. A., and Seymour, L. W. (1996) Atomic force microscopic analysis of the influence of the molecular weight of poly(L)lysine on the size of polyelectrolyte complexes formed with DNA. *Gene Ther. 3*, 269−273.
(20) Golan, R., Pietrasanta, L. I., Hsieh, W., and Hansma, H. C. (1999) DNA toroids: Stages in condensation. *Biochemistry 38*, 14069−14076.
(21) Perales, J. C., Grossmann, G. A., Molas, M., Liu, C., Ferkol, T., Harpst, J., Oda, H., and Hanson, R. W. (1997) Biochemical and functional characterization of DNA complexes capable of targeting genes to hepatocytes via the asialoglycoprotein receptor. *J. Biol. Chem. 272*, 7398−7407.
(22) Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R., and Wagner, E. (1998) The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. *Gene Ther. 5*, 1425−1433.
(23) Derrien, D., Midoux, P., Petit, C., Nègre, E., Mayer, R., Monsigny, M., and Roche, A. C. (1989) Muramyl dipeptide bound to poly-L-lysine substituted with mannose and gluconoyl residues as macrophage activators. *Glycoconjugate J. 6*, 241−255.
(24) Brasier, A. R., Tate, J. E., and Habener, J. F. (1989) Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *BioTechniques 7*, 1116−1123.
(25) Kunzelmann, K., Schwiebert, E. M., Zeitlin, P. L., Kuo, W.-L., Stanton, B. A., and Gruenert, D. C. (1993) An immortalized cystic fibrosis tracheal epithelial cell line homozygous for the ΔF508 CFTR mutation. *Am. J. Respir. Cell Mol. Biol. 8*, 522−529.
(26) Chen, T. R. (1977) In situ detection of mycoplasma contamination in cell line cultures by fluorescent Hoeschst 33258 stain. *Exp. Cell Res. 104*, 255−262.
(27) Monsigny, M., Roche, A. C., and Midoux, P. (1984) Uptake of neoglycoproteins via membrane lectin(s) of L1210 cells evidenced by quantitative flow cytofluorometry an drug targeting. *Biol. Cell 51*, 187−196.
(28) Midoux, P., Roche, A. C., and Monsigny, M. (1987) Quantitation of the binding, uptake and degradation of fluoresceinylated neoglycoproteins by flow cytometry. *Cytometry 8*, 327−334.
(29) De Wet, J. R., Wood, K. V., De Luca, M., Helinsky, D. R., and Subramani, S. (1987) Firefly luciferase gene structure and expression in mammalian cells. *Mol. Cell. Biol. 7*, 725−737.
(30) Meadows, D. H., Jardetzky, O., Epand, R. M., Ruterjans, H. H., and Scheraga, H. A. (1968) Assignment of the histidine peaks in the nuclear magnetic resonance spectrum of ribonuclease. *Proc. Natl. Acad. Sci. U.S.A. 60*, 766−772.
(31) Dash, P R., Read, M. L., Fisher, K. D., Howard, K. A., Wolfert, M., Oupicky, D., Subr, V., Strohalm, J., Ulrich, K., and Seymour, L. W. (2000) Decreased binding to proteins and cells of polymeric gene delivery vectors surface modified with a multivalent hydrophilic polymer and retargeting through attachment of transferrin. *J. Biol. Cell 275*, 3793−3802.
(32) Dash, P. R., Read, M. L., Barrett, L. B., Wolfert, M. A., and Seymour, L. W. (1999) Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. *Gene Ther. 6*, 643−650.
(33) Ogris, M., Brunner, S., Schuller, S., Kircheis, R., and Wagner, E. (1999) PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther. 6*, 595−605.
(34) Mislich, K. A., and Baldeschwieler, J. D. (1996) Evidence for the role of proteoglycans in cation-mediated gene transfert. *Proc. Natl. Acad. Sci. U.S.A. 93*, 12349−12354.
(35) Plank, C., Mechter, K., Szoka, F., and Wagner, E. (1996) Activation of the complement system by synthetic DNA complexes: A potential barrier for intravenous gene delivery. *Hum. Gene Ther. 7*, 1437−1446.
(36) Burckhardt, C., Zimmer, C., and Luck, G. (1976) Conformation and reactivity of DNA in the complex with proteins. III. Helix-coil transition and conformational studies of model complexes of DNA's with poly-L-histidine. *Nucleic Acids Res. 3*, 537−559.
(37) Santella, R. M., and Li, H. J. (1977) Interaction between Poly(L-lysine^{48}, L-histidine^{52}) and DNA. *Biopolymers 16*, 1879−1894.
(38) Pichon, C., Bello Roufaï, M., Monsigny, M., and Midoux, P. (2000) Histidylated oligolysines increase the transmembrane passage and the biological activity of antisense oligonucleotides. *Nucleic Acid Res. 28*, 504−512.

BC0000738

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
09/297,519	05/03/99	MIDOUX	P 410.015

EXAMINER	
NGUYEN, D	
ART UNIT	**PAPER NUMBER**
1633	7

HM12/0816

CHARLES A MUSERLIAN
BIERMAN MUSERLIAN AND LUCAS
600 THIRD AVENUE
NEW YORK NY 10016

RECEIVED
AUG 18 2000

DATE MAILED: 08/16/00

BIERMAN, MUSERLIAN AND LUCAS

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Sep 16, 00
(+1) Oct 16
(+2) Nov 16
(+3) Dec 16
(+4) Jan 16, 01

PTO-90C (Rev. 2/95)

2 - Mail Copy

Office Action Summary

Application No.	Applicant(s)
09/297,519	Midoux et al.
Examiner	**Group Art Unit**
Dave Nguyen	1633

☐ Responsive to communication(s) filed on _____.

☐ This action is **FINAL.**

☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire 1 month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) 22-44 is/are pending in the application.

Of the above, claim(s) _____ is/are withdrawn from consideration.

☐ Claim(s) _____ is/are allowed.

☐ Claim(s) _____ is/are rejected.

☐ Claim(s) _____ is/are objected to.

☒ Claims 22-44 are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on _____ is ☐approved ☐disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) _____.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____.

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☐ Notice of References Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____

☐ Interview Summary, PTO-413

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

— *SEE OFFICE ACTION ON THE FOLLOWING PAGES* —

Serial Number: 09/297,519

Art Unit: 1633

Claims 20 and 21 have been amended; Claims 1-19 have been canceled; and claims 22-44 have been added by the preliminary amendment filed on July 23, 1999.

Election/Restriction

Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 20-39, drawn to a polymeric conjugate comprising substituted monomers having free NH_3^+ groups, methods of transfecting cells by using the polymeric conjugate as a carrier, are classified in class 435, 320.1, and subclass 455.

II. Claims 41 and 42, drawn to DNA vaccine against influenza, and method of using the vaccince to produce a protective effect in a warm-blooded animal from influenza, are classified in class 514, subclass 44.

III. Claim 43, are directed to a method of using a polymeric conjugate comprising substituted monomers having free NH_3^+ groups and a DNA encoding a corrective gene to treat any congenital or acquired metabolic deficiency in warmed blood animal, are classified in class 514, subclass 44.

IV. Claim 44, drawn to a method of using a polymeric conjugate comprising substituted monomers having free NH_3^+ groups and a DNA encoding an anti-tumor protein or product to treat a tumor in warm-blooded animals, are classified in class 514, subclass 44.

The inventions are distinct, each from the other because of the following reasons:

Invention I, and inventions II-IV are related as product and processes of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product (MPEP § 806.05(h)). In the instant case, polymeric carrier of Invention I are not limited for use in each of the DNA therapy claimed in Inventions II, III,

and IV, and can be clearly used in any other invention as set forth in Invention II, III, and IV. In addition, the polymeric carrier can also be used for enhancing gene delivery in cultured cells and in diagnostic assays.

Although there are no provisions under the section for "Relationship of Inventions" in MPEP 806.05 for inventive groups that are directed to different methods, restriction is deemed to be proper because these methods appear to constitute patentably distinct inventions for the following reasons: Inventions II, III, and IV are directed to materially different methods and distinct goals, *e.g.*, each invention of the respective group comprises materially distinct steps that would generate distinct functions and effects. For example, the invention I employs a DNA that is asserted to generate a therapeutic effect in the treatment of an influenza infection. However, the invention of Invention III, for example, is drawn to a gene therapy method of using any DNA that exhibits a therapeutic effect in the treatment of any congenital or acquired metabolic deficiency in any warm-blooded animal, wherein any administration route is employed.

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art because of their divergent subject matter, fall into different statutory classes of invention, and are separately classified and searched, it would be unduly burdensome for the examine to search and consider patentability of all the presently pending claims. Thus, restriction for examination purposes as indicated is proper.

Applicant is advised that the response to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 C.F.R. § 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a diligently-filed petition under 37 C.F.R. § 1.48(b) and by the fee required under 37 C.F.R. § 1.17(h).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to *Dave Nguyen* whose telephone number is **(703) 305-2024**.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, *John LeGuyader*, may be reached at **(703) 308-0447**.

Any inquiry of a general nature or relating to the status of this application should be directed to the *Group receptionist* whose telephone number is **(703) 304-0196**.

Dave Nguyen

Patent Examiner

Art Unit: 1633

Our Ref.: 410.015

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: :
Patrick MIDOUX et al :
Serial No.: 09/297,519 :
Filed: May 3, 1999 :
For: NOVEL...CELL MEMBRANES :

600 Third Avenue
New York, NY 10016
August 29, 2000

RESPONSE

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Responsive to the Office Action of August 16, 2000, Applicants request reconsideration of the application in view of the remarks presented herein.

The Examiner has required a four-way restriction requirement. Group I includes claims 20 to 39, Group II is claims 41 and 42, Group III is claim 43 and Group IV is claim 44. The claims were deemed independent for the reasons set forth in the Office Action.

Applicants elect claims 20 to 39 for prosecution and reserve the right to file a divisional application directed to non-elected claims 41 to 44.

Since the first Office Action was merely a restriction requirement and due to the fact that the application was filed on

May 3, 1999, a prompt examination on the merits is requested.

Respectfully submitted,
BIERMAN, MUSERLIAN AND LUCAS

Charles A. Muserlian, #19,683
Attorney for Applicant(s)
Tel. # (212) 661-8000

CAM:sd
Enclosure: Return Receipt Postcard

[USB 96 AP IDM HIS]

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.

EXAMINER	
ART UNIT	PAPER NUMBER

RECEIVED
DEC 15 2000

DATE MAILED:

BIERMAN, MUSERLIAN AND LUCAS

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

(3) Mar 13
(4) Apr 13
(5) May 13
(6) June 13, 01

Election/Restriction

Applicant's election without traverse of the Group I claims (claims 20-40) in the response filed on August 30, 2000 is acknowledged.

Claims 41-44 are withdrawn from further consideration by the examiner, 37 C.F.R. 1.142(b) as being drawn to nonelected inventions.

Elected claims 20-40 are pending for examination.

Applicant is advised that the information regarding the cross-reference to the as-filed application as the national phase application under 35 U.S.C. 371 and its claim of priority to foreign applications under 35 U.S.C. 119 (a)-(d) should be stated the first paragraph of the specification.

This application does not contain an abstract of the disclosure as required by 37 CFR 1.72(b). An abstract on a separate sheet is required.

For applicant's convenience, the following guidelines are cited to illustrate the preferred layout and content for patent applications. These guidelines are suggested for the applicant's use.

Arrangement of the Specification

The following order or arrangement is preferred in framing the specification and, except for the reference to a "Microfiche Appendix" and the drawings, each of the lettered items should appear in upper case, without underlining or bold type, as section headings. If no text follows the section heading, the phrase "Not Applicable" should follow the section heading:

(a) Title of the Invention.
(b) Cross - Reference to Related Applications.
(c) Statement Regarding Federally Sponsored Research or Development.
(d) Reference to a "Microfiche Appendix" (see 37 CFR 1.96).
(e) Background of the Invention.
    1. Field of the Invention.
    2. Description of Related Art including information disclosed under 37 CFR 1.97 and 1.98.
(f) Brief Summary of the Invention.
(g) Brief Description of the Several Views of the Drawing(s).
(h) Detailed Description of the Invention.
(i) Claim or Claims (commencing on a separate sheet).
(j) Abstract of the Disclosure (commencing on a separate sheet).
(k) Drawings.

(I) Sequence Listing (see 37 CFR 1.821 - 1.825).

Foreign documents, WO 92/13570, PCT written Opinion with respect to PCT/FR97/02022, FR 2.107.756, EP 0387775, EP 0388758, FR 2 719 316 submitted with the IDS of record have been considered only to the extent possible without an English Translation.

Sequence Rules

This application contains sequence disclosures (see page 20, and claim 33, for example) that are encompassed by the definitions for nucleotide and/or amino acid sequences set forth in 37 CFR 1.821(a)(1) and (a)(2). However, this application fails to comply with the requirements of 37 CFR 1.821 through 1.825 for the reason(s) set forth on the attached Notice To Comply With Requirements For Patent Applications Containing Nucleotide Sequence And/Or Amino Acid Sequence Disclosures.

The following is a quotation of the first paragraph of 35 U.S.C. § 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 21-40, at best understood, are readable on a polymeric complex comprising of DNA and a polymeric conjugate which comprises an unspecified polymer formed from monomers having free NH3+ groups, wherein at least 10% of which are substituted by unspecified residues that must exhibit a biological function of being protonated in a weakly acid medium causing destabilization of cell membrane, said unspecified residues also carrying an unspecified functional group that must exhibit a biological function of not being active with recognition signal recognized by a cell membrane receptor, and wherein some of the free NH3+ are substituted by non-charged residues having at least one-OH which must exhibit a biological function of not being active with the recognition signal recognized by a cell membrane receptor, which in turn may be substituted by unspecified cellular recognition signals, are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed,

sequence, oligosaccharide based recognition signal or monosaccharide based recognition signal.

With respect to claims readable on a genus of certain fragments of anti-inflammatory peptides, the specification discloses no specific or "certain fragments" as claimed.

With respect to claims readable on a genus of "antagonists" of peptide hormones, the specification discloses no specific antagonists or a representative number of species of antagonists as claimed.

With respect to claims readable on a genus of "ribozymes" as therapeutic DNA, the specification discloses no specific ribozymes and/or a representative number of species of antagonists as claimed.

In other words, it is apparent that on the basis of applicant's disclosure, an adequate written description of the invention defined by the claims requires more than a mere statement that it is part of the invention and reference to potential methods and/or assays for making the polymer genus as claimed; what is required is the knowledge in the prior art and/or a description as to the availability of a representative number of species of polymeric conjugates and/or functional groups thereof that must exhibit the disclosed biological functions as contemplated by the as-filed specification.

It is not sufficient to support the present claimed invention by disclosing polymeric complexes comprising the polymer with the formula as set forth in claim 34, wherein R is a residue with an imidazole nucleus, or a compound having the formulae as set forth on pages 12 and 13 of the as-filed specification, said polymer further conjugated to gluconyl based non-charged residues, because disclosure of no more than that, as in the instant case, is simply a wish to know the identity of any and/or all other polymeric conjugates having other residues with the biological functions as contemplated by the specification and the claims. The claimed invention as a whole is not adequately described if the claims require essential or critical elements which are not adequately described in the specification and which is not conventional in the art as of applicants effective filing date. Claiming all polymeric conjugates and/or functional groups and/or therapeutic DNA thereof that must possess the biological properties as contemplated by applicant's disclosure without defining what means will do so is not in compliance with the written description requirement. Rather, it is an attempt to preempt the future before it has arrived. (See *Fiers v. Revel*, 25 USPQ2d 1601 (CA FC 1993) and *Regents of the Univ. Calif. v. Eli Lilly & Co.*, 43 USPQ2d 1398 (CA FC,

1997)). Possession may be shown by actual reduction to practice, clear depiction of the invention in a detailed drawing, or by describing the invention with sufficient relevant identifying characteristics such that a person skilled in the art would recognize that the inventor had possession of the claimed invention. Pfaff v. Wells Electronics, Inc., 48 USPQ2d 1641, 1646 (1998). The skilled artisan cannot envision the detailed structure of a genus of the claimed polymeric complexes that must exhibit the contemplated biological functions, and therefore, conception is not achieved until reduction to practice has occurred, regardless of the complexity or simplicity of the structures and/or methods disclosed in the as-filed specification. Thus, In view of the reasons set forth above, one skilled in the art at the time the invention was made would not have recognized that applicant was in possession of the claimed invention as presently claimed.

Claims 21-40 are rejected under 35 U.S.C. 112, first paragraph, because the specification is enabling only for claims limited to:

DNA/polymeric complexes comprising the polymer with the formula as set forth in claim 34, wherein R is a residue with an imidazole nucleus, or a compound having the formulae as set forth on pages 12 and 13 of the as-filed specification, said polymer further conjugated to gluconyl based non-charged residues, wherein the recognition signals are peptide based, oligosaccharide based, or monosaccharide based recognition signals, which complexes exhibit the biological functions as disclosed in the base claim 22.

The specification does not reasonably provide enablement for the presently pending claims encompassing any and/or all other polymeric delivery vectors including those that embrace therapeutic applications as recited in the claims. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Factors to be considered in determining whether a disclosure would require undue experimentation have been summarized in In re Wands, 858 F.2d 731, 8USPQ2d 1400 (Fed. Cir. 1988). They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the

presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

Specifically, since the claimed invention is not supported by a sufficient written description (for possessing of the genus of polymeric complexes as recited in the claims, particularly in view of the reasons set forth above, one skilled in the art would not known how to use and make the claimed invention so that it would operate as intended, *e.g.* functions as a nucleic acid delivery vector that exhibits all of the biological functions as recited in the claimed invention.

Furthermore, it appears that the only intended use of the claimed polymeric complexes is for enhancing delivery of nucleic acid molecules into a target cell so as to generate a more effective transmembrane passage of nucleic acid molecules as compared to well-known unsubstituted polylysine/DNA complexes and other substituted polymeric vectors having agents which reduce the number of charges on the polymeric vector. However, the state of the art exemplified by Verma, Nature, Vol. 389, pages 239-242, 1997, indicates:

"The Achilles hell of gene therapy is gene delivery, and this the aspect that we will concentrate on here. Thus far, the problem has been an inability to deliver genes efficiently and to obtain sustained expression. There are two categories of delivery vehicle ('vector'). The first comprises the non-viral vectors, ranging from direct injection of DNA to mixing the DNA with polylysine or cationic lipids that allow the gene to cross the cell membrane. Most of these approaches suffer from poor efficiency of delivery and transient expression of the gene" (page 239, column 3).

Given that transient gene expression remains a major obstacle of gene transfer methods of using polylysine based vectors, as stated in Verma, and given that the claimed invention, *e.g.,* claims 21 and 34, for example, encompasses therapeutic applications of the DNA/polymeric complexes, it is not apparent as to how one skilled in the art determines, without undue experimentation, which of the disclosed gene therapy vector of polymeric complexes are effective for use as intended therapeutic complexes. Furthermore, it is apparent that only intended use of claimed polymeric complexes comprising a target cell

recognition signal from the as-filed disclosure is to use the complexes for targeted *in vivo* delivery of nucleic acids to any target cell. However, major considerations for any gene transfer or gene therapy protocol involve issues such as amount of DNA constructs to be administered, what amount is considered to be therapeutically effective for all of the claimed nucleic acid molecules, the route and time course of administration, the sites of administration, successful uptake of the claimed DNA at the target site, expression of the DNA at the target site in amounts of effecting the treatment in a treated subject (Anderson, Nature, Vol. 392, pp. 25-30, April 1998). More specifically, Anderson teaches that results in one particular animal model have not always reflected what happens in another animal model (page 28, column 1, first paragraph), that gene therapy is a powerful new technology that still requires several years before it will make a noticeable impact on the treatment of disease, and that several major deficiencies still exist including poor delivery systems, both viral and non-viral, and poor gene expression after genes are delivered (page 30, column 1, last paragraph). Anderson further teaches that the reason for the low efficiency of gene transfer and expression in human patients is that we still lack a basis understanding of how vectors should be constructed, what regulatory sequences are appropriated for which cell types. Verma *et al.* further state that "although there are reagents that increase the efficiency of delivery, transient expression of the transgene is a conceptual hurdle that needs to be addresses" (page 239, column 3, first paragraph). Furthermore, Verma *et al.* indicates that factors including the nature of the diseases and/or disorders, the nature of a DNA and/or target tissue, and a delivery system and/or amounts of the DNA complexes employed in the delivery system that would generate a therapeutic effect *in vivo* must be considered for any gene therapy method to be successful (page 238, columns 1 and 2).

With respect to the use of therapeutic DNA as claimed in claim 34 or as encompassed the *in vivo* gene therapy method claims including antisense, ribozymes, triplex oligonucleotides to treat a tissue *in vivo* therapeutically, the application does not demonstrate a therapeutic effect in any subject using any of the disclosed DNA as claimed. More specific as to the state of the art of antisense therapy, Branch (TIBS 23, pp. 45-50, 1998) even in 19998, antisense and ribozyme therapy remains unpredictable (entire document).

There is no factual evidence from the as-filed application of any *in vivo* beneficial affect generated

from any targeted gene transfer vector as claimed. The specification does not provide sufficient guidance and/or factual evidence demonstrating a reasonable correlation between the disclosure and the subject matter being sought in the claims. Thus, it is not apparent as to how one skilled in the art reasonably extrapolates, without any undue experimentation, from *in vitro* use of polymeric complexes comprising polylysine conjugated with residues comprising an imidazole nucleus and an NH3+, and gluconyl based non-charged residues to any and/or all other claimed polymer complexes that embrace therapeutic applications as contemplated by the application. Thus, the specification is further not enabling under 35 U.S.C. 112, first paragraph, for any and/or therapeutic nucleic acid constructs within the context of treatment of any disease in any subject, particularly on the basis of applicant's disclosure and the reasons stated in the art of record.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 20-40 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 20 is indefinite because the claim is dependent on a canceled claim, claim 12, thereby rendering the claim incomplete, *e.g.,* see MPEP 608.01(n)(V).

Claims 22, 24, 25, 36 and claims dependent therefrom are indefinite in the recitation of the phrase "and optionally containing a molecule ....by substitution of some of the free NH3 of the monomer...or some of the non-charged residues....or on some of the residues causing.....or on some the residues causing a destabilization of cell membranes by substitution of the optional free NH3+" because the phrase appears to be Markush-type claims , but do not appear to use accepted Markush language (MPEP 2173.05(h)(a)). In addition, the phrase as a whole is vague and does not point out the intended scope of the placement of the recognition signal in the polymeric complex as claimed, particularly since the incorporation of the "or on" renders the claim unclear as to its metes and bounds of the

placement of the recognition signal. In addition, the phrase "are not active with the recognition signal" is indefinite because the word "active" is relative in meanings and does not point out the intended scope of the claims. It is not apparent as to how the "are not active" occurs physically between the claimed residue and the recognition signal.. The term "the recognition signal" also lacks an antecedent basis because it is well-known in the art that there are plurality of recognition signal peptides, for example, that bind to a cell membrane receptor. The phrase "the recognition signal required by a cell membrane receptor" is also indefinite because it is not apparent as to how the "required" identifies the intended scope of the "recognition signal".

Claim 29 is also indefinite in the recitation of "and optionally by a molecule having a recognition signal" because it is not apparent as to what is exactly meant by "and optionally by". It is not apparent as to is exactly the active step that links structurally the "optionally by". In addition, the "recognition signal" is indefinite because it is not apparent as to is exactly the metes and bounds of the term, nor is it apparent as to what is exactly the materials that are recognized by the "signals". In addition, the phrase "10 to 90% of R being free ...optionally substituted 0 to 50%" is vague and confusing because it is not apparent as to what is exactly the meaning of the "optionally substituted 0 to 50%", particularly since the phase appears to miss a preposition word and is not grammatically correct.

Claim 30 is indefinite in the recitation of "R" because the base claims 26 and 27 do not recite any R. However, the claim states that R is in the complex of claim 27.

Claim 31 is indefinite in the recitation of "wherein m is 2 to 7 -NH-CO-(CH2OH)m-R1 and is selected from the group" is grammatically incorrect and thus, it is not apparent as to what is exactly the intended scope of the claim.

In claim 33, step a), the phrase "certain of their fragments" are indefinite because the phrase is relative in meanings and does not identify as to what exactly constitutes the relative meaning of "certain".

In claim 33, step C), the "certain cells" is indefinite because the term is relative in meanings and does not identify as to what exactly constitute the relative meaning of "certain".

Claim 34 is indefinite because the claim attempts to recite nucleic acid as "protein", "enzymes", or disease names, particularly since a nucleic acid or genes are not *per se* proteins, enzymes, or disease names. The claim should be amended to clarify the language by indicating, for example, that "gene coding from luciferase" instead of "genes containing luciferase", that "gene encoding factor VIII and IX" instead of "genes with a therapeutic purpose

selected from the group consisting of hypercholesterolaemia, ...". In addition, the phrase "genes which code for ribozymes" is definite because a ribozyme is not a gene that codes for a mRNA but rather a synthetic nucleic acid molecule. In addition, the term "such as" is indefinite because the term does not define the intended scope of the claim.

Claim 35 is indefinite in the recitation of "the lysine units" because the term is not present in the base claim 22, and thus, the term lacks an antecedent basis.

Claim 36 is indefinite in the recitation of "the above-mentioned polymer" because the term is not present in any where above the recitation in the claim, and thus, the term lacks an antecedent basis. In addition, the "monomer units being at least 10% by residues causing" is grammatically incorrect, and thus rendering the claim vague and confusing. Appropriate correction is required.

Claim 39 is indefinite in the recitation of "...and/or..." because the recitation fails to clearly define as to what is exactly the intended scope of the claim. The claim is also indefinite in the recitation of "corresponding" because the term is relative in meanings and does not define the intended scope "the protein".

In claim 40, the phrase "carrying a recognition signal being a function of a target cell optionally bonded beforehand to the polymer conjugate" is indefinite because it is not apparent as to how the signal is structurally linked to a polymeric conjugate of claim 36. In addition, it is not apparent as to which of the polymeric conjugate of claim 36 is intended to be claimed in claim 40 in view of the recitation of "a polymeric conjugate of claim 36". In addition, it is not apparent as how the "signal being a function of a target cell" is exactly to be meant. What is exactly the function applicant intends to define as a functional limitation for the recited "signal"? In addition, the term "reagent" as recited numerously in the claim and defined by only functional limitations are indefinite because it is not apparent as to what is exactly the metes and bounds of the "reagents". In addition, the term "system" is also indefinite because it is not apparent as to what is exactly the material(s) that constitute the "system" having the recited function.

Claim 27 is objected because "he" on line is a misspelling of "the".

Claim Rejections - 35 U.S.C. § 103

The following is a quotation of 35 U.S.C. ' 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made. Subject matter developed by another person, which qualifies as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

To the extent that the claims, as best understood given numerous indefinite recitations present in the claims, are readable on a DNA/polymer complexes comprising a polylysine conjugated to a residue with an imidazole nucleus, said polymer further conjugated to gluconyl based non-charged residues, wherein the recognition signals are peptide based, oligosaccharide based, or monosaccharide based recognition signals, which complexes exhibit the biological functions as disclosed in the base claim 22, and are readable on *in vitro* gene transfer methods of using the complexes, the following rejection is applicable.

Claims 21-40 are rejected under 35 U.S.C. 103 as being unpatentable over either FR-A-2719316 (D1, cited in the written opinion from PCT officers) or Midoux *et al.* (US Pat No. 5,733,762, 3/1998, wherein Erbacher and Roche-Degremont constitute as another inventive entity, entire document), taken with Wang *et al.* (D3, cited in the Written Opinion from PCT officers).

D1 and Midoux describe a complex between at least one negatively charged nucleic acid and at least one positively charged polymeric conjugate bonded by electrostatic interaction. The polymeric conjugate contains a polymer of monomeric units with free NH3+ groups. The free NH3+ are substituted, with a ratio of at least 10%, by gluconyl based non-charge residues and do not bind to any recognition signal recognized by a cell membrane receptor (entire documents).

The difference between D1 or Midoux and the subject matter of the present claimed invention is that the claimed invention is directed to histidine residues that are protonable in a weakly acidic medium and further comprise a functional group enabling them to be bound to the polymer while not being recognized by a cell membrane receptor. The objective of employing histidine residues or residues with an

imidazole nucleus conjugated to polylysine, for example, is to enhance the protection of transfectinng nucleic acid from lysosomal decomposition following endocytosis.

However, at the time the invention was made, D3 describes the fusion-mediating properties of polyhistidine relative to liposomes. The concept of fusion is caused by the polycationic nature of polyhistidine having an acid pH, and to the combination of the polycation with membrane phospholipids that induces phase separation in the dual lipid layer (D3, abstract). D3 also indicates that the fusion-mediating behavior associated with polyhistidine having a low pH is more effective than the one associated with Ca2+ or polylysine. More specifically, D3 indicates that a charge ratio of only 0.2 or less between the polyhistidine and the liposome enables effective fusion to be ensured, whereas it must exceed 0.7 with Ca2+ and be of around 1 with polylysine (see page 4414, column 2, last sentence to page 4415, column 1, line 13; table IV). In addition and most importantly, D3 suggests that if the interaction between the hydrophobic segments of viral envelope glycoproteins is an important step in the fusion process, the protonation of the histidine residues of the viral protein with an acidic pH would be an alternative fusion means (page 4115, last paragraph).

It would have been obvious for one of ordinary skill in the art to have incorporated histidine residues to any of the free NH3 groups of the polylysine in D1 in order to enhance the fusion and translocation of DNA complexed with polylysine. One of ordinary skill in the art would have been motivated to have incorporated histidine residues to any of the free NH3 groups of the polylysine in D1 because of the reasons set forth in the preceding paragraphs.

To the extent that the claims are readable on specific substitution ratios, and further optional incorporation of cell-recognition peptides, it would have been obvious to one of ordinary skill in the art as a matter of design choice to employ any ratio and/or well known cell recognition peptides in the polymeric complexes of D1 taken with D3, particularly since such teachings are also disclosed in D1 (claims 1 and 4) and in Midoux *et al.*

To the extent that the claim are readable on residues belonging to the family of compounds that comprise an imidazole ring, having residues that are alkylimidazoles, the claims are directed to minor

modification and/or obvious variants of the polymeric complexes, and one of ordinary skill in the art would have been motivated as a matter of design choice to employ these well-known compounds as obvious variants of histidines, particularly since D1 teaches that the polymer includes a grouping of formula (I) and (II) (see D1, claims 6 and 8). Likewise, the selection of recognition signals, the selection of nucleic acids and the selection of the defining parameters of the polymer, *e.g.,* the substitution ration of the free NH3+ of the lysine units, the selection of the molecular weight of the nuclei acid and the average number of base pairs of the nucleic acid per monomeric unit molecule, are minor modification or options that a person of ordinary skill in the art would have been motivated to have as a matter of design choice, depending on each particular case (see D1, claims 11-13; and Midoux *et al.,* column 3-16). Thus, in the absence of unexpected results, the claims are obvious variants of one another.

Thus, the claimed invention as a whole was *prima facie* obvious.

Double Patenting Rejection

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970);and, *In re Thorington,* 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 21-40 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 9-15 of U.S. Patent No. 5,733,762, 3/1998, claims 9-15, and further in view of D3. Although the conflicting claims are not identical, they are not patentably distinct from each other because both sets of claims are readable on

a complex between at least one negatively charged nucleic acid and at least one positively charged

polymeric conjugate bonded by electrostatic interaction. The polymeric conjugate contains a polymer of monomeric units with free NH3+ groups. The free NH3+ are substituted, with a ratio of at least 10%, by gluconyl based non-charge residues and do not bind to any recognition signal recognized by a cell membrane receptor.

The difference between Midoux and the subject matter of the present claimed invention is that the claimed invention is directed to histidine residues that are protonable in a weakly acidic medium and further comprise a functional group enabling them to be bound to the polymer while not being recognized by a cell membrane receptor. The objective of employing histidine residues or residues with an imidazole nucleus conjugated to polylysine, for example, is to enhance the protection of transfectinng nucleic acid from lysosomal decomposition following endocytosis.

However, at the time the invention was made, D3 describes the fusion-mediating properties of polyhistidine relative to liposomes. The concept of fusion is caused by the polycationic nature of polyhistidine having an acid pH, and to the combination of the polycation with membrane phospholipids that induces phase separation in the dual lipid layer (D3, abstract). D3 also indicates that the fusion-mediating behavior associated with polyhistidine having a low pH is more effective than the one associated with Ca2+ or polylysine. More specifically, D3 indicates that a charge ratio of only 0.2 or less between the polyhistidine and the liposome enables effective fusion to be ensured, whereas it must exceed 0.7 with Ca2+ and be of around 1 with polylysine (see page 4414, column 2, last sentence to page 4415, column 1, line 13; table IV). In addition and most importantly, D3 suggests that if the interaction between the hydrophobic segments of viral envelope glycoproteins is an important step in the fusion process, the protonation of the histidine residues of the viral protein with an acidic pH would be an alternative fusion means (page 4115, last paragraph).

It would have been obvious to one of ordinary skill in the art to have incorporated histidine residues to any of the free NH3 groups of the polylysine in D1 in order to enhance the fusion and translocation of DNA complexed with polylysine. One of ordinary skill in the art would have been motivated to have incorporated histidine residues to any of the free NH3 groups of the polylysine in D1 because of the reasons

set forth in the preceding paragraphs. Thus, the subject mater as claimed in this instant application, wherein histidine residues are incorporated to NH3+ groups of the polylysine polymer, is obvious variants of the subject matter as recited in claims 9-15 of the '762 patent.

To the extent that the claims are readable on specific substitution ratios, and further optional incorporation of cell-recognition peptides, it would have been obvious to one of ordinary skill in the art as a matter of design choice to employ any ratio and/or well known cell recognition peptides in the polymeric complexes of Midoux taken with D3, particularly since such teachings are also disclosed in the claims of Midoux (claim 9).

To the extent that the claim are readable on recognition signals, the selection of nucleic acids and the selection of the defining parameters of the polymer, *e.g.*, the substitution ration of the free NH3+ of the lysine units, the selection of the molecular weight of the nuclei acid and the average number of base pairs of the nucleic acid per monomeric unit molecule, are minor modification or options that a person of ordinary skill in the art would have been motivated to have as a matter of design choice, depending on each particular case (see claims 9-15). Thus, in the absence of unexpected results, the claims are obvious variants of one another.

No claims are allowed.

Any inquiry concerning this communication or earlier communications regarding the formalities should be directed to Patent Analyst Kimberly Davis, whose telephone number is **(703) 308-0009**.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to examiner *Dave Nguyen* whose telephone number is **(703) 305-2024**.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, *Deborah Clark*, may be reached at **(703) 305-4051.**

Any inquiry of a general nature or relating to the status of this application should be directed to the *Group receptionist* whose telephone number is **(703) 308-0196**.

Dave Nguyen
Patent Examiner
Art Unit: 1633

Application No.: 09/297,519

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

Applicant must file the items indicated below within the time period set the Office action to which the Notice is attached to avoid abandonment under 35 U.S.C. § 133 (extensions of time may be obtained under the provisions of 37 CFR 1.136(a)).

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

- [X] 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990). If the effective filing date is on or after July 1, 1998, see the final rulemaking notice published at 63 FR 29620 (June 1, 1998) and 1211 OG 82 (June 23, 1998).
- [X] 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).
- [X] 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).
- [ ] 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing."
- [ ] 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).
- [ ] 6. The paper copy of the "Sequence Listing" is not the same as the computer readable from of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).
- [ ] 7. Other: _

Applicant Must Provide:

- [X] An initial or substitute computer readable form (CRF) copy of the "Sequence Listing".
- [X] An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification.
- [X] A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d).

For questions regarding compliance to these requirements, please contact:

For Rules Interpretation, call (703) 308-4216
For CRF Submission Help, call (703) 308-4212
PatentIn Software Program Support
Technical Assistance.....703-287-0200
To Purchase PatentIn Software.....703-306-2600

PLEASE RETURN A COPY OF THIS NOTICE WITH YOUR REPLY

Notice of References Cited

Application/Control No.	Applicant(s)/Patent Under Reexamination
09/297,519	MIDOUX ET AL.
Examiner	**Art Unit**
Dave Nguyen	1633

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUBCLASS	DOCUMENT SOURCE ** APS	DOCUMENT SOURCE ** OTHER
☐	A	5,977,084	Nov. 1999	Szoka, Jr. et al.	514	44	☐	☐
☐	B	6,030,941	Feb. 2000	Summerton et al.	514	2	☐	☐
☐	C	5,846,530	Dec. 1998	Soon-Shiong et al.	424	93.7	☐	☐
☐	D	5,733,762	Mar. 1998	Midoux et al.	435	172.3	☐	☐
☐	E						☐	☐
☐	F						☐	☐
☐	G						☐	☐
☐	H						☐	☐
☐	I						☐	☐
☐	J						☐	☐
☐	K						☐	☐
☐	L						☐	☐
☐	M						☐	☐

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS	DOCUMENT SOURCE ** APS	DOCUMENT SOURCE ** OTHER
☐	N	95/30020	Nov. 1995	WO--	Midoux et al.	-	--	☐	☐
☐	O							☐	☐
☐	P							☐	☐
☐	Q							☐	☐
☐	R							☐	☐
☐	S							☐	☐
☐	T							☐	☐

NON-PATENT DOCUMENTS

*		DOCUMENT (Including Author, Title Date, Source, and Pertinent Pages)	DOCUMENT SOURCE ** APS	DOCUMENT SOURCE ** OTHER
☐	U	Anderson, Human gene therapy, Nature, Vol. 392, pages 25-30	☐	☐
☐	V	Verma et al., Gene therapy-promises, problems and prospects, Nature, Vol. 389, pages 239-242	☐	☐
☐	W	Branch, A good antisense molecule is hard to find, TIBS, Feb. 23, 1998, pages 45-50	☐	☐
		Erbacher et al., Glycosylated Polylysine/DNA Complexes: Gene Transfer Efficiency in Relation with the		

Notice of References Cited	Application/Control No. 09/297,519	Applicant(s)/Patent Under Reexamination MIDOUX ET AL.	
	Examiner Dave Nguyen	Art Unit 1633	Page 2 of 2

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUBCLASS	DOCUMENT SOURCE ** APS	OTHER
☐	A						☐	☐
☐	B						☐	☐
☐	C						☐	☐
☐	D						☐	☐
☐	E						☐	☐
☐	F						☐	☐
☐	G						☐	☐
☐	H						☐	☐
☐	I						☐	☐
☐	J						☐	☐
☐	K						☐	☐
☐	L						☐	☐
☐	M						☐	☐

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS	DOCUMENT SOURCE ** APS	OTHER
☐	N							☐	☐
☐	O							☐	☐
☐	P							☐	☐
☐	Q							☐	☐
☐	R							☐	☐
☐	S							☐	☐
☐	T							☐	☐

NON-PATENT DOCUMENTS

*		DOCUMENT (Including Author, Title Date, Source, and Pertinent Pages)	DOCUMENT SOURCE ** APS	OTHER
☐	U	Erbacher et al., Gene Transfer by DNA/Glycosylated Polylsine Complexes into Human Blood Macrophages, Human Gene Therapy, Vol. 7, April 10, 1996, pages 721-729	☐	☐
☐	V	Kollen et al., Gluconoylated and Glycosylated Polylysines As Vector for Gene Transfer on Cystic Fibrosis Airways Epithelial Cells, Human Gene Therapy, Vol. 7, August 20, 1996, pages 1577-1586	☐	☐
☐	W		☐	☐

09/297519

AH #1 [redacted] 03 MAY 1999

PTO/SB/08 (11-98)

Sheet 1 of 2

Form PTO-1449	Docket Number (Optional)	Application Number
INFORMATION DISCLOSURE CITATION IN AN APPLICATION	410.015	PCT/FR97/02022
(Use several sheets if necessary)	Applicant: MIDOUX et al	
	Filing Date: November 10, 1997	Group Art Unit

U. S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
DV	5 16 632 0	11/24/92	Wu et al	530	395	—

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	Translation YES	Translation NO
DV	2 71 931 6	11/3/95	France	—	—		X
	9 21 357 0	08/20/92	WO	—	—		X
	0 38 777 5	9/19/90	EPO	—	—		X
	0 38 875	8/26/90	EPO	—	—		X
DV	9 21 103 7	7/9/92	WO	—	—	X	

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

DV	Mezo et al, "Carrier...Poly(L-Lysine)", Biopolymers, Vol. 33, No. 6, 1993, pp. 873-885, XP002034603
	Wang et al, "Polyhistidine...Liposomes", Biochemistry, Vol. 23 No. 19, 1984, pp. 4409-4416, XP002016041
DV	Midoux et al, "Specific...Cells", Nucleic Acids Research, Vol. 21, No.4, February 25, 1993, pp. 871-878, XP000371764

EXAMINER	DATE CONSIDERED
Dan	11/30/00

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP § 609. Draw line through

09/2975

5[illegible] 3 MAY

09/257,519

PTO/SB/08 (11-[illegible])

Sheet 2 of 2

Form PTO-1449

INFORMATION DISCLOSURE CITATION IN AN APPLICATION

(Use several sheets if necessary)

Docket Number (Optional)	Application Number
410.015	PCT/FR97/02022
Applicant	
MIDOUX et al	
Filing Date	Group Art Unit
November 10, 1997	1633

U. S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	Translation YES	Translation NO
DL	2 10 77 56	5/5/72	France				
✓	0 35 02 46	1/10/90	EPO				X
						X	

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

EXAMINER	DATE CONSIDERED
Dal[illegible]	11/30/00

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP [illegible]

Form PTO 948 (Rev. 8-98) U.S. DEPARTMENT OF COMMERCE - Patent and Trademark Office Appl. No. 9/297,579

NOTICE OF DRAFTSPERSON'S PATENT DRAWING REVIEW

The drawing(s) filed (insert date) 5-3-99 are:

A. ☐ approved by the Draftsperson under 37 CFR 1.84 or 1.152.

B. ☑ objected to by the Draftsperson under 37 CFR 1.84 or 1.152 for the reasons indicated below. The Examiner will require submission of new, corrected drawings when necessary. Corrected drawing sheets must be submitted according to the instructions on the back of this notice.

1. DRAWINGS. 37 CFR 1.84(a): Acceptable categories of drawings: Black ink. Color.
   - ___ Color drawings are not acceptable until petiton is granted. Fig(s) _____
   - ___ Pencil and non black ink not permitted. Fig(s) _____
2. PHOTOGRAPHS. 37 CFR 1.84 (b)
   - ___ 1 full-tone set is required. Fig(s) _____ ystol board or
   - ___ Photographs not properly mounted (must _____ photographic double-weight paper). F___
   - ___ Poor quality (half-tone). Fig(s) _____
3. TYPE OF PAPER. 37 CFR 1.84(e) id durable.
   - ___ Paper not flexible, strong, w
   - Fig(s) _____ itings, interlineations,
   - ___ Erasures, alterations, ks not accepted. Fig(s) _____
   - folds, copy machin not acceptable (too thin).
   - ___ Mylar, velum p
   - Fig(s) ___ / CFR 1.84(f): Acceptable sizes:
4. SIZE OF PAPE 9.7 cm (DIN size A4)
   - ___ 21.0 cby 27.9 cm (8 1/2 x 11 inches)
   - ___ 21 awing sheets not the same size.
   - ___ eet(s) _____
   - Drawings sheets not an acceptable size. Fig(s) _____

ARGINS. 37 CFR 1.84(g): Acceptable margins:

Top 2.5 cm Left 2.5cm Right 1.5 cm Bottom 1.0 cm
SIZE: A4 Size

Top 2.5 cm Left 2.5 cm Right 1.5 cm Bottom 1.0 cm
SIZE: 8 1/2 x 11

Margins not acceptable. Fig(s) 1-5 9

_____ Top (T) _____ Left (L)

_____ Right (R) _____ Bottom (B)

6. VIEWS. 37 CFR 1.84(h)

REMINDER: Specification may require revision to correspond to drawing changes.

Partial views. 37 CFR 1.84(h)(2)

___ Brackets needed to show figure as one entity.

8. ARRANGEMENT OF VIEWS. 37 CFR 1.84(i)
   - ___ Words do not appear on a horizontal, left-to-right fashion when page is either upright or turned so that the top becomes the right side, except for graphs. Fig(s) _____
9. SCALE. 37 CFR 1.84(k)
   - ___ Scale not large enough to show mechanism without crowding when drawing is reduced in size to two-thirds in reproduction. Fig(s) _____
10. CHARACTER OF LINES, NUMBERS, & LETTERS. 37 CFR 1.84(i)
    - ___ Lines, numbers & letters not uniformly thick and well defined, clean, durable, and black (poor line quality). Fig(s) 1-5 9
11. SHADING. 37 CFR 1.84(m)
    - ___ Solid black areas pale. Fig(s) _____
    - ___ Solid black shading not permitted. Fig(s) _____
    - ___ Shade lines, pale, rough and blurred. Fig(s) _____
12. NUMBERS, LETTERS, & REFERENCE CHARACTERS. 37 CFR 1.84(p)
    - ___ Numbers and reference characters not plain and legible. Fig(s) 1-5 9
    - ___ Figure legends are poor. Fig(s) _____
    - ___ Numbers and reference characters not oriented in the same direction as the view. 37 CFR 1.84(p)(1) Fig(s) _____
    - ___ English alphabet not used. 37 CFR 1.84(p)(2) Figs _____
    - ___ Numbers, letters and reference characters must be at least .32 cm (1/8 inch) in height. 37 CFR 1.84(p)(3) Fig(s) _____
13. LEAD LINES. 37 CFR 1.84(q)
    - ___ Lead lines cross each other. Fig(s) _____
    - ___ Lead lines missing. Fig(s) _____
14. NUMBERING OF SHEETS OF DRAWINGS. 37 CFR 1.84(t)
    - ___ Sheets not numbered consecutively, and in Arabic numerals beginning with number 1. Sheet(s) _____
15. NUMBERING OF VIEWS. 37 CFR 1.84(u)

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
09/227,619	02/03/99	MOUDJX	P 311,017

HM22/0118

CHARLES A MUSERLIAN
BIERMAN MUSERLIAN AND LUCAS
600 THIRD AVENUE
NEW YORK NY 10016

EXAMINER	
NGUYEN,D	
ART UNIT	**PAPER NUMBER**
1638	10

RECEIVED
JAN 22 2001

DATE MAILED: [illegible]

BIERMAN, MUSERLIAN AND LUCAS

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

(3) Apr 18
(4) May 18
(5) June 18
(6) July 18, 01

Office Action Summary	Application No.	Applicant(s)	
	09/297,519	MIDOUX ET AL.	
	Examiner	Art Unit	
	Christopher Drabik	1633	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136 (a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *20 August 2000* .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *20-44* is/are pending in the application.

4a) Of the above claim(s) *41-44* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *20-40* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claims _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. & 119(e).

Attachment(s)

15)☒ Notice of References Cited (PTO-892)

18)☐ Interview Summary (PTO-413) Paper No(s). ____

In response to applicant's telephone inquiry of regarding the last Office action, the following corrective action is taken.

Copies of the references cited in the PTO-982 not previously supplied are now enclosed:

The period for response of 3 months set in the Office action dated December 13, 2000 is restarted to begin with the dated of this Office action.

Election/Restriction

Applicant's election without traverse of the Group I claims (claims 20-40) in the response filed on August 30, 2000 is acknowledged.

Claims 41-44 are withdrawn from further consideration by the examiner, 37 C.F.R. 1.142(b) as being drawn to nonelected inventions.

Elected claims 20-40 are pending for examination.

Applicant is advised that the information regarding the cross-reference to the as-filed application as the national phase application under 35 U.S.C. 371 and its claim of priority to foreign applications under 35 U.S.C. 119 (a)-(d) should be stated the first paragraph of the specification.

This application does not contain an abstract of the disclosure as required by 37 CFR 1.72(b). An abstract on a separate sheet is required.

For applicant's convenience, the following guidelines are cited to illustrate the preferred layout and content for patent applications. These guidelines are suggested for the applicant's use.

Arrangement of the Specification

The following order or arrangement is preferred in framing the specification and, except for the reference to a "Microfiche Appendix" and the drawings, each of the lettered items should appear in upper case, without underlining or bold type, as section headings. If no text follows the section heading, the phrase "Not Applicable" should follow the section heading:

(a) Title of the Invention.
(b) Cross - Reference to Related Applications.
(c) Statement Regarding Federally Sponsored Research or Development.
(d) Reference to a "Microfiche Appendix" (see 37 CFR 1.96).

(e) Background of the Invention.
1. Field of the Invention.
2. Description of Related Art including information disclosed under 37 CFR 1.97 and 1.98.

(f) Brief Summary of the Invention.
(g) Brief Description of the Several Views of the Drawing(s).
(h) Detailed Description of the Invention.
(i) Claim or Claims (commencing on a separate sheet).
(j) Abstract of the Disclosure (commencing on a separate sheet).
(k) Drawings.
(l) Sequence Listing (see 37 CFR 1.821 - 1.825).

Foreign documents, WO 92/13570, PCT written Opinion with respect to PCT/FR97/02022, FR 2.107.756, EP 0387775, EP 0388758, FR 2 719 316 submitted with the IDS of record have been considered only to the extent possible without an English Translation.

Sequence Rules

This application contains sequence disclosures (see page 20, and claim 33, for example) that are encompassed by the definitions for nucleotide and/or amino acid sequences set forth in 37 CFR 1.821(a)(1) and (a)(2). However, this application fails to comply with the requirements of 37 CFR 1.821 through 1.825 for the reason(s) set forth on the attached Notice To Comply With Requirements For Patent Applications Containing Nucleotide Sequence And/Or Amino Acid Sequence Disclosures.

The following is a quotation of the first paragraph of 35 U.S.C. § 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 21-40, at best understood, are readable on a polymeric complex comprising of DNA and a polymeric conjugate which comprises an unspecified polymer formed from monomers having free NH3+ groups, wherein at least 10% of which are substituted by unspecified residues that must exhibit a biological function of being protonated in a weakly acid medium causing destabilization of cell membrane, said unspecified residues also carrying an unspecified functional group that must exhibit a biological function of not being active with recognition signal recognized by a cell membrane receptor, and wherein some of the

free NH3+ are substituted by non-charged residues having at least one-OH which must exhibit a biological function of not being active with the recognition signal recognized by a cell membrane receptor, which in turn may be substituted by unspecified cellular recognition signals, are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The specification and the state of the prior art of record provide sufficient description of polymeric complexes comprising the polymer with the formula as set forth in claim 34, wherein R is a residue with an imidazole nucleus, or a compound having the formulae as set forth on pages 12 and 13 of the as-filed specification, said polymer further conjugated to gluconyl based non-charged residues.

With respect to claims readable on a genus of polymers formed from monomers having free NH3+ groups which must exhibit a biological function of being able to function as a conjugate to other disclosed functional groups, *e.g.,* nucleic acid molecules, residues comprising an imidazole nucleus and an NH3+, gluconyl based non-charged residues, and cellular recognition signals, and to function as a whole as a nucleic acid transfer vector, the specification only provides sufficient description of the polymer with the formula as set forth in claim 29 or 34.

With respect to claims readable on a genus of unspecified residues that must exhibit a biological function of being protonated in a weakly acid medium and causing destabilization of cell membrane, said unspecified residues also carrying an unspecified functional group that must exhibit a biological function of not being active with recognition signal recognized by a cell membrane receptor, the specification only provides sufficient description of a polylysine based polymeric complex conjugated to residues having an imidazole nucleus and an NH3+ functional group, and residues having the formulae as set forth on pages 12 and 13 of the as-filed specification

With respect to claims readable on a genus of non-charged residues having at least one-OH which must exhibit a biological function of not being active with the recognition signal recognized by a cell membrane receptor, which in turn may be substituted by unspecified cellular recognition signals, the

specification only provides sufficient description of a polysine based polymeric complex conjugated to residues having an imidazole nucleus and an NH3+ functional group and to gluconyl based non-charged residues.

With respect to claims readable on a genus of cellular recognition signals, the specification only provides sufficient description of cellular recognition signals which are peptide based recognition signal sequence, oligosaccharide based recognition signal or monosaccharide based recognition signal.

With respect to claims readable on a genus of certain fragments of anti-inflammatory peptides, the specification discloses no specific or "certain fragments" as claimed.

With respect to claims readable on a genus of "antagonists" of peptide hormones, the specification discloses no specific antagonists or a representative number of species of antagonists as claimed.

With respect to claims readable on a genus of "ribozymes" as therapeutic DNA, the specification discloses no specific ribozymes and/or a representative number of species of antagonists as claimed.

In other words, it is apparent that on the basis of applicant's disclosure, an adequate written description of the invention defined by the claims requires more than a mere statement that it is part of the invention and reference to potential methods and/or assays for making the polymer genus as claimed; what is required is the knowledge in the prior art and/or a description as to the availability of a representative number of species of polymeric conjugates and/or functional groups thereof that must exhibit the disclosed biological functions as contemplated by the as-filed specification.

It is not sufficient to support the present claimed invention by disclosing polymeric complexes comprising the polymer with the formula as set forth in claim 34, wherein R is a residue with an imidazole nucleus, or a compound having the formulae as set forth on pages 12 and 13 of the as-filed specification, said polymer further conjugated to gluconyl based non-charged residues, because disclosure of no more than that, as in the instant case, is simply a wish to know the identity of any and/or all other polymeric conjugates having other residues with the biological functions as contemplated by the specification and the claims. The claimed invention as a whole is not adequately described if the claims require essential or critical elements which are not adequately described in the specification and which is not conventional in the

art as of applicants effective filing date. Claiming all polymeric conjugates and/or functional groups and/or therapeutic DNA thereof that must possess the biological properties as contemplated by applicant's disclosure without defining what means will do so is not in compliance with the written description requirement. Rather, it is an attempt to preempt the future before it has arrived. (See *Fiers v. Revel*, 25 USPQ2d 1601 (CA FC 1993) and *Regents of the Univ. Calif. v. Eli Lilly & Co.*, 43 USPQ2d 1398 (CA FC, 1997)). Possession may be shown by actual reduction to practice, clear depiction of the invention in a detailed drawing, or by describing the invention with sufficient relevant identifying characteristics such that a person skilled in the art would recognize that the inventor had possession of the claimed invention. Pfaff v. Wells Electronics, Inc., 48 USPQ2d 1641, 1646 (1998). The skilled artisan cannot envision the detailed structure of a genus of the claimed polymeric complexes that must exhibit the contemplated biological functions, and therefore, conception is not achieved until reduction to practice has occurred, regardless of the complexity or simplicity of the structures and/or methods disclosed in the as-filed specification. Thus, In view of the reasons set forth above, one skilled in the art at the time the invention was made would not have recognized that applicant was in possession of the claimed invention as presently claimed.

Claims 21-40 are rejected under 35 U.S.C. 112, first paragraph, because the specification is enabling only for claims limited to:

DNA/polymeric complexes comprising the polymer with the formula as set forth in claim 34, wherein R is a residue with an imidazole nucleus, or a compound having the formulae as set forth on pages 12 and 13 of the as-filed specification, said polymer further conjugated to gluconyl based non-charged residues, wherein the recognition signals are peptide based, oligosaccharide based, or monosaccharide based recognition signals, which complexes exhibit the biological functions as disclosed in the base claim 22.

The specification does not reasonably provide enablement for the presently pending claims encompassing any and/or all other polymeric delivery vectors including those that embrace therapeutic applications as recited in the claims. The specification does not enable any person skilled in the art to

which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Factors to be considered in determining whether a disclosure would require undue experimentation have been summarized in In re Wands, 858 F.2d 731, 8USPQ2d 1400 (Fed. Cir. 1988). They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

Specifically, since the claimed invention is not supported by a sufficient written description (for possessing of the genus of polymeric complexes as recited in the claims, particularly in view of the reasons set forth above, one skilled in the art would not known how to use and make the claimed invention so that it would operate as intended, *e.g.* functions as a nucleic acid delivery vector that exhibits all of the biological functions as recited in the claimed invention.

Furthermore, it appears that the only intended use of the claimed polymeric complexes is for enhancing delivery of nucleic acid molecules into a target cell so as to generate a more effective transmembrane passage of nucleic acid molecules as compared to well-known unsubstituted polylysine/DNA complexes and other substituted polymeric vectors having agents which reduce the number of charges on the polymeric vector. However, the state of the art exemplified by Verma, Nature, Vol. 389, pages 239-242, 1997, indicates:

"The Achilles hell of gene therapy is gene delivery, and this the aspect that we will concentrate on here. Thus far, the problem has been an inability to deliver genes efficiently and to obtain sustained expression. There are two categories of delivery vehicle ('vector'). The first comprises the non-viral vectors, ranging from direct injection of DNA to mixing the DNA with polylysine or cationic lipids that allow the gene to cross the cell membrane. Most of these approaches suffer from poor efficiency of delivery and transient expression of the gene" (page 239, column 3).

Given that transient gene expression remains a major obstacle of gene transfer methods of using

polylysine based vectors, as stated in Verma, and given that the claimed invention, *e.g.*, claims 21 and 34, for example, encompasses therapeutic applications of the DNA/polymeric complexes, it is not apparent as to how one skilled in the art determines, without undue experimentation, which of the disclosed gene therapy vector of polymeric complexes are effective for use as intended therapeutic complexes. Furthermore, it is apparent that only intended use of claimed polymeric complexes comprising a target cell recognition signal from the as-filed disclosure is to use the complexes for targeted *in vivo* delivery of nucleic acids to any target cell. However, major considerations for any gene transfer or gene therapy protocol involve issues such as amount of DNA constructs to be administered, what amount is considered to be therapeutically effective for all of the claimed nucleic acid molecules, the route and time course of administration, the sites of administration, successful uptake of the claimed DNA at the target site, expression of the DNA at the target site in amounts of effecting the treatment in a treated subject (Anderson, Nature, Vol. 392, pp. 25-30, April 1998). More specifically, Anderson teaches that results in one particular animal model have not always reflected what happens in another animal model (page 28, column 1, first paragraph), that gene therapy is a powerful new technology that still requires several years before it will make a noticeable impact on the treatment of disease, and that several major deficiencies still exist including poor delivery systems, both viral and non-viral, and poor gene expression after genes are delivered (page 30, column 1, last paragraph). Anderson further teaches that the reason for the low efficiency of gene transfer and expression in human patients is that we still lack a basis understanding of how vectors should be constructed, what regulatory sequences are appropriated for which cell types. Verma *et al.* further state that "although there are reagents that increase the efficiency of delivery, transient expression of the transgene is a conceptual hurdle that needs to be addresses" (page 239, column 3, first paragraph). Furthermore, Verma *et al.* indicates that factors including the nature of the diseases and/or disorders, the nature of a DNA and/or target tissue, and a delivery system and/or amounts of the DNA complexes employed in the delivery system that would generate a therapeutic effect *in vivo* must be considered for any gene therapy method to be successful (page 238, columns 1 and 2).

With respect to the use of therapeutic DNA as claimed in claim 34 or as encompassed the *in vivo*

gene therapy method claims including antisense, ribozymes, triplex oligonucleotides to treat a tissue *in vivo* therapeutically, the application does not demonstrate a therapeutic effect in any subject using any of the disclosed DNA as claimed. More specific as to the state of the art of antisense therapy, Branch (TIBS 23, pp. 45-50, 1998) even in 19998, antisense and ribozyme therapy remains unpredictable (entire document).

There is no factual evidence from the as-filed application of any *in vivo* beneficial affect generated from any targeted gene transfer vector as claimed. The specification does not provide sufficient guidance and/or factual evidence demonstrating a reasonable correlation between the disclosure and the subject matter being sought in the claims. Thus, it is not apparent as to how one skilled in the art reasonably extrapolates, without any undue experimentation, from *in vitro* use of polymeric complexes comprising polylysine conjugated with residues comprising an imidazole nucleus and an NH3+, and gluconyl based non-charged residues to any and/or all other claimed polymer complexes that embrace therapeutic applications as contemplated by the application. Thus, the specification is further not enabling under 35 U.S.C. 112, first paragraph, for any and/or therapeutic nucleic acid constructs within the context of treatment of any disease in any subject, particularly on the basis of applicant's disclosure and the reasons stated in the art of record.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 20-40 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 20 is indefinite because the claim is dependent on a canceled claim, claim 12, thereby rendering the claim incomplete, *e.g.,* see MPEP 608.01(n)(V).

Claims 22, 24, 25, 36 and claims dependent therefrom are indefinite in the recitation of the phrase "and optionally containing a molecule ....by substitution of some of the free NH3 of the monomer...or some of the non-

charged residues....or on some of the residues causing.....or on some the residues causing a destabilization of cell membranes by substitution of the optional free NH3+" because the phrase appears to be Markush-type claims , but do not appear to use accepted Markush language (MPEP 2173.05(h)(a)). In addition, the phrase as a whole is vague and does not point out the intended scope of the placement of the recognition signal in the polymeric complex as claimed, particularly since the incorporation of the "or on" renders the claim unclear as to its metes and bounds of the placement of the recognition signal. In addition, the phrase "are not active with the recognition signal" is indefinite because the word "active" is relative in meanings and does not point out the intended scope of the claims. It is not apparent as to how the "are not active" occurs physically between the claimed residue and the recognition signal.. The term "the recognition signal" also lacks an antecedent basis because it is well-known in the art that there are plurality of recognition signal peptides, for example, that bind to a cell membrane receptor. The phrase "the recognition signal required by a cell membrane receptor" is also indefinite because it is not apparent as to how the "required" identifies the intended scope of the "recognition signal".

Claim 29 is also indefinite in the recitation of "and optionally by a molecule having a recognition signal" because it is not apparent as to what is exactly meant by "and optionally by". It is not apparent as to is exactly the active step that links structurally the "optionally by". In addition, the "recognition signal" is indefinite because it is not apparent as to is exactly the metes and bounds of the term, nor is it apparent as to what is exactly the materials that are recognized by the "signals". In addition, the phrase "10 to 90% of R being free ...optionally substituted 0 to 50%" is vague and confusing because it is not apparent as to what is exactly the meaning of the "optionally substituted 0 to 50%", particularly since the phase appears to miss a preposition word and is not grammatically correct.

Claim 30 is indefinite in the recitation of "R" because the base claims 26 and 27 do not recite any R. However, the claim states that R is in the complex of claim 27.

Claim 31 is indefinite in the recitation of "wherein m is 2 to 7 -NH-CO-(CH2OH)m-R1 and is selected from the group" is grammatically incorrect and thus, it is not apparent as to what is exactly the intended scope of the claim.

In claim 33, step a), the phrase "certain of their fragments" are indefinite because the phrase is relative in meanings and does not identify as to what exactly constitutes the relative meaning of "certain".

In claim 33, step C), the "certain cells" is indefinite because the term is relative in meanings and does not identify as to what exactly constitute the relative meaning of "certain".

Claim 34 is indefinite because the claim attempts to recite nucleic acid as "protein", "enzymes", or disease names, particularly since a nucleic acid or genes are not *per se* proteins, enzymes, or disease names. The claim should be amended to clarify the language by indicating, for example, that "gene coding from luciferase" instead of "genes containing luciferase", that "gene encoding factor VIII and IX" instead of "genes with a therapeutic purpose selected from the group consisting of hypercholesterolaemia, ...". In addition, the phrase "genes which code for ribozymes" is definite because a ribozyme is not a gene that codes for a mRNA but rather a synthetic nucleic acid molecule. In addition, the term "such as" is indefinite because the term does not define the intended scope of the claim.

Claim 35 is indefinite in the recitation of "the lysine units" because the term is not present in the base claim 22, and thus, the term lacks an antecedent basis.

Claim 36 is indefinite in the recitation of "the above-mentioned polymer" because the term is not present in any where above the recitation in the claim, and thus, the term lacks an antecedent basis. In addition, the "monomer units being at least 10% by residues causing" is grammatically incorrect, and thus rendering the claim vague and confusing. Appropriate correction is required.

Claim 39 is indefinite in the recitation of "...and/or..." because the recitation fails to clearly define as to what is exactly the intended scope of the claim. The claim is also indefinite in the recitation of "corresponding" because the term is relative in meanings and does not define the intended scope "the protein".

In claim 40, the phrase "carrying a recognition signal being a function of a target cell optionally bonded beforehand to the polymer conjugate" is indefinite because it is not apparent as to how the signal is structurally linked to a polymeric conjugate of claim 36. In addition, it is not apparent as to which of the polymeric conjugate of claim 36 is intended to be claimed in claim 40 in view of the recitation of "a polymeric conjugate of claim 36". In addition, it is not apparent as how the "signal being a function of a target cell" is exactly to be meant. What is exactly the function applicant intends to define as a functional limitation for the recited "signal"? In addition, the term "reagent" as recited numerously in the claim and defined by only functional limitations are indefinite because it is not apparent

as to what is exactly the metes and bounds of the "reagents". In addition, the term "system" is also indefinite because it is not apparent as to what is exactly the material(s) that constitute the "system" having the recited function.

Claim 27 is objected because "he" on line is a misspelling of "the".

Claim Rejections - 35 U.S.C. § 103

The following is a quotation of 35 U.S.C. ' 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made. Subject matter developed by another person, which qualifies as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

To the extent that the claims, as best understood given numerous indefinite recitations present in the claims, are readable on a DNA/polymer complexes comprising a polylysine conjugated to a residue with an imidazole nucleus, said polymer further conjugated to gluconyl based non-charged residues, wherein the recognition signals are peptide based, oligosaccharide based, or monosaccharide based recognition signals, which complexes exhibit the biological functions as disclosed in the base claim 22, and are readable on *in vitro* gene transfer methods of using the complexes, the following rejection is applicable.

Claims 21-40 are rejected under 35 U.S.C. 103 as being unpatentable over either FR-A-2719316 (D1, cited in the written opinion from PCT officers) or Midoux *et al.* (US Pat No. 5,733,762, 3/1998, wherein Erbacher and Roche-Degremont constitute as another inventive entity, entire document), taken with Wang *et al.* (D3, cited in the Written Opinion from PCT officers).

D1 and Midoux describe a complex between at least one negatively charged nucleic acid and at least one positively charged polymeric conjugate bonded by electrostatic interaction. The polymeric conjugate contains a polymer of monomeric units with free NH3+ groups. The free NH3+ are substituted,

with a ratio of at least 10%, by gluconyl based non-charge residues and do not bind to any recognition signal recognized by a cell membrane receptor (entire documents).

The difference between D1 or Midoux and the subject matter of the present claimed invention is that the claimed invention is directed to histidine residues that are protonable in a weakly acidic medium and further comprise a functional group enabling them to be bound to the polymer while not being recognized by a cell membrane receptor. The objective of employing histidine residues or residues with an imidazole nucleus conjugated to polylysine, for example, is to enhance the protection of transfectinng nucleic acid from lysosomal decomposition following endocytosis.

However, at the time the invention was made, D3 describes the fusion-mediating properties of polyhistidine relative to liposomes. The concept of fusion is caused by the polycationic nature of polyhistidine having an acid pH, and to the combination of the polycation with membrane phospholipids that induces phase separation in the dual lipid layer (D3, abstract). D3 also indicates that the fusion-mediating behavior associated with polyhistidine having a low pH is more effective than the one associated with Ca2+ or polylysine. More specifically, D3 indicates that a charge ratio of only 0.2 or less between the polyhistidine and the liposome enables effective fusion to be ensured, whereas it must exceed 0.7 with Ca2+ and be of around 1 with polylysine (see page 4414, column 2, last sentence to page 4415, column 1, line 13; table IV). In addition and most importantly, D3 suggests that if the interaction between the hydrophobic segments of viral envelope glycoproteins is an important step in the fusion process, the protonation of the histidine residues of the viral protein with an acidic pH would be an alternative fusion means (page 4115, last paragraph).

It would have been obvious for one of ordinary skill in the art to have incorporated histidine residues to any of the free NH3 groups of the polylysine in D1 in order to enhance the fusion and translocation of DNA complexed with polylysine. One of ordinary skill in the art would have been motivated to have incorporated histidine residues to any of the free NH3 groups of the polylysine in D1 because of the reasons set forth in the preceding paragraphs.

To the extent that the claims are readable on specific substitution ratios, and further optional

incorporation of cell-recognition peptides, it would have been obvious to one of ordinary skill in the art as a matter of design choice to employ any ratio and/or well known cell recognition peptides in the polymeric complexes of D1 taken with D3, particularly since such teachings are also disclosed in D1 (claims 1 and 4) and in Midoux *et al.*

To the extent that the claim are readable on residues belonging to the family of compounds that comprise an imidazole ring, having residues that are alkylimidazoles, the claims are directed to minor modification and/or obvious variants of the polymeric complexes, and one of ordinary skill in the art would have been motivated as a matter of design choice to employ these well-known compounds as obvious variants of histidines, particularly since D1 teaches that the polymer includes a grouping of formula (I) and (II) (see D1, claims 6 and 8). Likewise, the selection of recognition signals, the selection of nucleic acids and the selection of the defining parameters of the polymer, *e.g.,* the substitution ratio of the free NH3+ of the lysine units, the selection of the molecular weight of the nuclei acid and the average number of base pairs of the nucleic acid per monomeric unit molecule, are minor modification or options that a person of ordinary skill in the art would have been motivated to have as a matter of design choice, depending on each particular case (see D1, claims 11-13; and Midoux *et al.,* column 3-16). Thus, in the absence of unexpected results, the claims are obvious variants of one another.

Thus, the claimed invention as a whole was *prima facie* obvious.

Double Patenting Rejection

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970);and, *In re Thorington,* 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer.

A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 21-40 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 9-15 of U.S. Patent No. 5,733,762, 3/1998, claims 9-15, and further in view of D3. Although the conflicting claims are not identical, they are not patentably distinct from each other because both sets of claims are readable on

a complex between at least one negatively charged nucleic acid and at least one positively charged polymeric conjugate bonded by electrostatic interaction. The polymeric conjugate contains a polymer of monomeric units with free NH3+ groups. The free NH3+ are substituted, with a ratio of at least 10%, by gluconyl based non-charge residues and do not bind to any recognition signal recognized by a cell membrane receptor.

The difference between Midoux and the subject matter of the present claimed invention is that the claimed invention is directed to histidine residues that are protonable in a weakly acidic medium and further comprise a functional group enabling them to be bound to the polymer while not being recognized by a cell membrane receptor. The objective of employing histidine residues or residues with an imidazole nucleus conjugated to polylysine, for example, is to enhance the protection of transfectinng nucleic acid from lysosomal decomposition following endocytosis.

However, at the time the invention was made, D3 describes the fusion-mediating properties of polyhistidine relative to liposomes. The concept of fusion is caused by the polycationic nature of polyhistidine having an acid pH, and to the combination of the polycation with membrane phospholipids that induces phase separation in the dual lipid layer (D3, abstract). D3 also indicates that the fusion-mediating behavior associated with polyhistidine having a low pH is more effective than the one associated with Ca2+ or polylysine. More specifically, D3 indicates that a charge ratio of only 0.2 or less between the polyhistidine and the liposome enables effective fusion to be ensured, whereas it must exceed 0.7 with Ca2+ and be of around 1 with polylysine (see page 4414, column 2, last sentence to page 4415, column 1, line 13; table IV). In addition and most importantly, D3 suggests that if the interaction between the hydrophobic segments of viral envelope glycoproteins is an important step in the fusion process, the protonation of the histidine

residues of the viral protein with an acidic pH would be an alternative fusion means (page 4115, last paragraph).

It would have been obvious to one of ordinary skill in the art to have incorporated histidine residues to any of the free NH3 groups of the polylysine in D1 in order to enhance the fusion and translocation of DNA complexed with polylysine. One of ordinary skill in the art would have been motivated to have incorporated histidine residues to any of the free NH3 groups of the polylysine in D1 because of the reasons set forth in the preceding paragraphs. Thus, the subject mater as claimed in this instant application, wherein histidine residues are incorporated to NH3+ groups of the polylysine polymer, is obvious variants of the subject matter as recited in claims 9-15 of the '762 patent.

To the extent that the claims are readable on specific substitution ratios, and further optional incorporation of cell-recognition peptides, it would have been obvious to one of ordinary skill in the art as a matter of design choice to employ any ratio and/or well known cell recognition peptides in the polymeric complexes of Midoux taken with D3, particularly since such teachings are also disclosed in the claims of Midoux (claim 9).

To the extent that the claim are readable on recognition signals, the selection of nucleic acids and the selection of the defining parameters of the polymer, *e.g.,* the substitution ratio of the free NH3+ of the lysine units, the selection of the molecular weight of the nuclei acid and the average number of base pairs of the nucleic acid per monomeric unit molecule, are minor modification or options that a person of ordinary skill in the art would have been motivated to have as a matter of design choice, depending on each particular case (see claims 9-15). Thus, in the absence of unexpected results, the claims are obvious variants of one another.

No claims are allowed.

Any inquiry concerning this communication or earlier communications regarding the formalities should be directed to Patent Analyst Kimberly Davis, whose telephone number is **(703) 305-3015**.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to examiner *Dave Nguyen* whose telephone number is **(703) 305-2024**.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, *Deborah Clark*, may be reached at **(703) 305-4051.**

Any inquiry of a general nature or relating to the status of this application should be directed to the *Group receptionist* whose telephone number is **(703) 308-0196.**

Dave Nguyen

Patent Examiner

Art Unit: 1633

Notice of References Cited	Application/Control No. 09/297,519	Applicant(s)/Patent Under Reexamination MIDOUX ET AL.
	Examiner Dave Nguyen	Art Unit 1633
		Page 1 of 2

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUBCLASS	DOCUMENT SOURCE ** APS	OTHER
☐	A	5,977,084	Nov. 1999	Szoka, Jr. et al.	514	44	☐	☐
☐	B	6,030,941	Feb. 2000	Summerton et al.	514	2	☐	☐
☐	C	5,846,530	Dec. 1998	Soon-Shiong et al.	424	93.7	☐	☐
☐	D	5,733,762	Mar. 1998	Midoux et al.	435	172.3	☐	☐
☐	E						☐	☐
☐	F						☐	☐
☐	G						☐	☐
☐	H						☐	☐
☐	I						☐	☐
☐	J						☐	☐
☐	K						☐	☐
☐	L						☐	☐
☐	M						☐	☐

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS	DOCUMENT SOURCE ** APS	OTHER
☐	N	95/30020	Nov. 1995	WO--	Midoux et al.	-	--	☐	☐
☐	O							☐	☐
☐	P							☐	☐
☐	Q							☐	☐
☐	R							☐	☐
☐	S							☐	☐
☐	T							☐	☐

NON-PATENT DOCUMENTS

*		DOCUMENT (Including Author, Title Date, Source, and Pertinent Pages)	DOCUMENT SOURCE ** APS	OTHER
☐	U	Anderson, Human gene therapy, Nature, Vol. 392, pages 25-30	☐	☐
☐	V	Verma et al., Gene therapy-promises, problems and prospects, Nature, Vol. 389, pages 239-242	☐	☐
☐	W	Branch, A good antisense molecule is hard to find, TIBS, Feb. 23, 1998, pages 45-50	☐	☐
☐	X	Erbacher et al., Glycosylated Polylysine/DNA Complexes: Gene Transfer Efficiency in Relation with the Size and the Sugar Substitution Level of Glycosylated Polylysine	☐	☐

Notice of References Cited	Application/Control No. 09/297,519	Applicant(s)/Patent Under Reexamination MIDOUX ET AL.
	Examiner Dave Nguyen	Art Unit 1633
		Page 2 of 2

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUBCLASS	DOCUMENT SOURCE ** APS	OTHER
☐	A						☐	☐
☐	B						☐	☐
☐	C						☐	☐
☐	D						☐	☐
☐	E						☐	☐
☐	F						☐	☐
☐	G						☐	☐
☐	H						☐	☐
☐	I						☐	☐
☐	J						☐	☐
☐	K						☐	☐
☐	L						☐	☐
☐	M						☐	☐

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS	DOCUMENT SOURCE ** APS	OTHER
☐	N							☐	☐
☐	O							☐	☐
☐	P							☐	☐
☐	Q							☐	☐
☐	R							☐	☐
☐	S							☐	☐
☐	T							☐	☐

NON-PATENT DOCUMENTS

*		DOCUMENT (Including Author, Title Date, Source, and Pertinent Pages)	DOCUMENT SOURCE ** APS	OTHER
☐	U	Erbacher et al., Gene Transfer by DNA/Glycosylated Polylsine Complexes into Human Blood Macrophages, Human Gene Therapy, Vol. 7, April 10, 1996, pages 721-729	☐	☐
☐	V	Kollen et al., Gluconoylated and Glycosylated Polylysines As Vector for Gene Transfer on Cystic Fibrosis Airways Epithelial Cells, Human Gene Therapy, Vol. 7, August 20, 1996, pages 1577-1586	☐	☐
☐	W		☐	☐
☐	X		☐	☐

Grosset-Fournier & Demachy

Propriété Industrielle

Grosset-Fournier & Demachy
Membre du G.I.E. PA & GD

Charles Demachy
Docteur en Pharmacie
diplômé du CEIPI
(brevets, marques, modèles)

Catherine Grosset-Fournier
Ingénieur ENSCP
Mandataire Européen
diplômée du CEIPI
(brevets, marques, modèles)

Isabelle Laemmel
Maîtrise de Chimie
diplômée du CEIPI
(brevets, marques, modèles)

Autres membres du G.I.E. :
Pontet & Allano

Sylvain Allano
Docteur ès Sciences
Professeur des Universités

Bernard Pontet
Ingénieur INSA
Conseil en Brevets d'Invention
Mandataire Européen
diplômé du CEIPI
(brevets, marques, modèles)

BIERMAN MUSERLIAN & LUCAS,
600 Third Avenue
New York, NY 10016
USA
Attention : M. C. Muserlian

FACSIMILE/30 pages

O/Ref. : USB 96 AP IDM HIS/cgf-il

Paris, March 15, 2001

Subject : US Patent Application N°09/297 519 – National phase of the International application n° PCT/FR97/02022 filed on November 10, 1997
Priority : France n°96 13390 filed on November 15, 1996
In the name of : IDM IMMUNO-DESIGNED MOLECULES
Inventors : Midoux et al.
For : « Novel polymeric complexes for the transfection of nucleic acids, with residues causing the destabilisation of cell membranes. »

Dear Chuck,

We refer to your letter of January 24, 2001 concerning an office action of January 18, 2001 received in the above application which supersedes the office action of December 13, 2000.

- You will find enclosed herewith our instructions to reply to the pending Official Action. These instructions comprise :

1) A new set of claims (see pages 1 to 10),

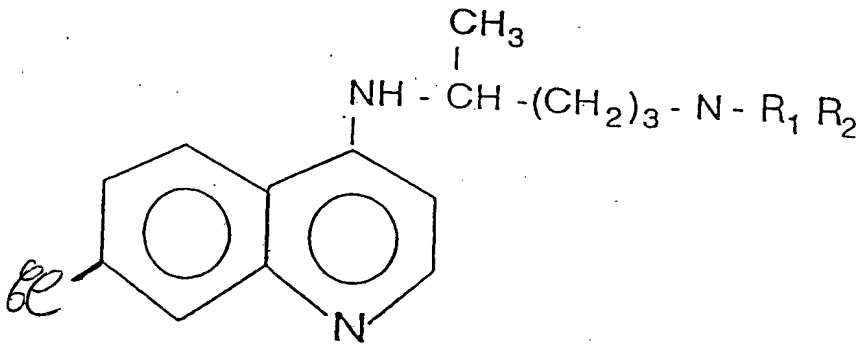
2) Experimental results added by the Applicant of the present invention in order to support the new set of claims and to overcome the Examiner's objections (see pages 11 to 17),

3) Inventive step of the present invention (see page 18),

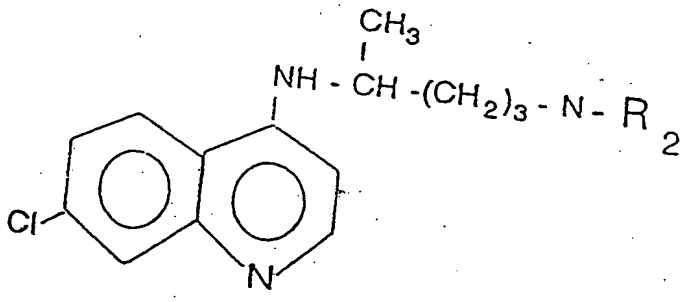
4) Figures 10 to 18 (9 pages) and,

5) Publication « Bello-Roufai and Midoux, 2001, Bioconjugate Chem. 12, 92-99 ».

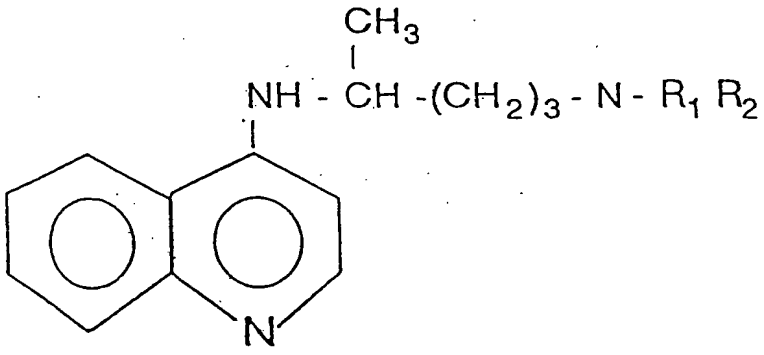
• Moreover, we draw your attention that there is a clerical error on pages 12 and 13 of the present invention regarding the formulae of quinolines. In fact the family of quinolines is represented by the following formula :



and not by :



or :



We leave it to you to correct this error.

We remain at your disposal for any information you may need.

Very truly yours

Catherine Grosset-Fournier

Encl : - our instructions to reply to the pending office action (27 pages),

- publication « Bello-Roufai and Midoux, 2001, Bioconjugate Chem. 12, 92-99 »

I) New set of claims

1. A complex comprised of at least one negatively charged nucleic acid and at least one positively charged polymeric conjugate with the bond therebetween being electrostatic in nature,

the polymeric conjugate containing a polylysine formed from monomers having free NH_3^+ groups,

at least 10% of the free NH_3^+ groups of the said polylysine are substituted by residues which can be protonated in a weakly acid medium causing destabilization of cell membranes,

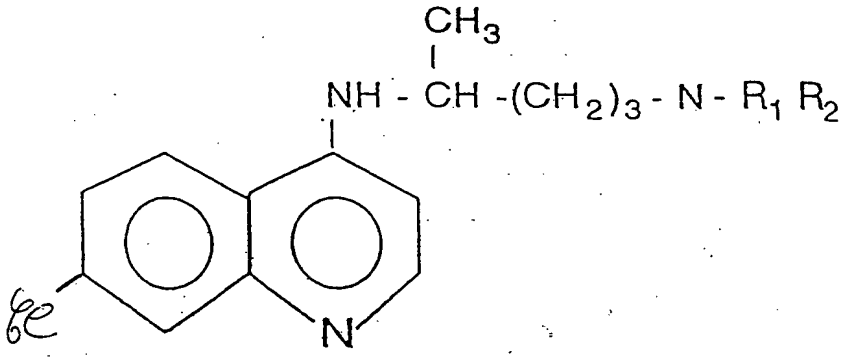
and optionally some of the free NH_3^+ groups of the said polylysine can be substituted by a molecule with a recognition signal recognized by a cell membrane receptor,

with the proviso that all the free NH_3^+ groups of the said polylysine make up at least 30% of the number of monomers of the skeleton of the polymeric conjugate,

wherein said residues causing destabilization of cell membranes in a weakly acid medium belong :

. to the family of compounds which carry an imidazole nucleus such as alkylimidazoles in which the alkyl radical contains 1 to 10 carbon atoms and in which only one of the nitrogen atoms of the imidazole nucleus is substituted, with said residues causing destabilization of cell membranes being more particularly selected from the group consisting of histidine, 4-carboxymethyl-imidazole, 3-(3-methyl-imidazol-4-yl)-alanine, (3-methyl-histidine), 3-(1-methyl-imidazol-4-yl)-alanine, (1-methyl-histidine), 2-carboxy-imidazole, histamine, 3-(imidazol-4-yl)-L-lactic acid, 2-(1-methyl-imidazol-4-yl)ethylamine, 2-(3-methyl-imidazol-4-yl)ethylamine, β-alanyl-histidine (carnosine), N-acetyl-histidine,

. to the family of quinolines of the formula :



in which R_1 is hydrogen, R_2 is $-(CH_2)_n-CO_2-H$ and n is an integer from 1 to 10,

with said quinolines being more particularly selected from the group consisting of 7-chloro-4-(amino-1-methylbutylamino)-quinoline, N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine, 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline (primaquine), N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine,

. to the family of pterines such as pteroic acid,

. to the family of pyridine such as nicotinic acid or quinolinic acid,

wherein said recognition signal is selected from the group consisting of :

. simple osides such as a monosaccharide selected from the group consisting of α or β conformers of 2-deoxy, of 2-amino or of 2-deoxy, 2-acetamido neutral monosaccharides ; α or β conformers of glycuronic acid derivatives of neutral monosaccharides ; α or β conformers of L-iduronic acid, of keto-deoxy-octonic acid, of N-acetyl-neuraminic acid, or of N-glycoloyl-neuraminic acid ; and α or β conformers of neutral 6-deoxy monosaccharides ;

or a disaccharide selected from the group consisting of lactose and mannopyranosylα-6mannopyranose,

or complex osides such as an oligosaccharide selected from the group consisting of $Lewis^a$, $Lewis^b$, $Lewis^x$, oligomannosides and oligolactosamines,

. peptides.

2. The complex of claim 1 wherein the free NH_3^+ groups of the polylysine are substituted with a non-charged residue causing a reduction in the positive charge of the polymeric conjugate which facilitates salting out of the nucleic acids upon dissociation of the complex, the said non-charged residue being a gluconyl.

3. The complex of claim 1 wherein said recognition signal is a peptide chosen from :

(a) anti-inflammatory peptides recognized by receptors of the vascular wall, such as :

- vasodilator intestinal polypeptide (VIP)

HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2

- atrial natriuretic polypeptide (ANP)

SLRRSSCFGGRMDRIGAQSGLGCNSFRY

- lipocortin

HDMNKVLDL

- bradykinin

RPPGFSPFR ;

(b) ligand peptides of integrins, such as peptides containing the sequence RGD, fibronectin ligand ;

(c) chemiotactic factors, such as formyl-peptides and their antagonists :

FMLP, (N-formyl-Met-Leu-Phe);

(d) peptide hormones, such as

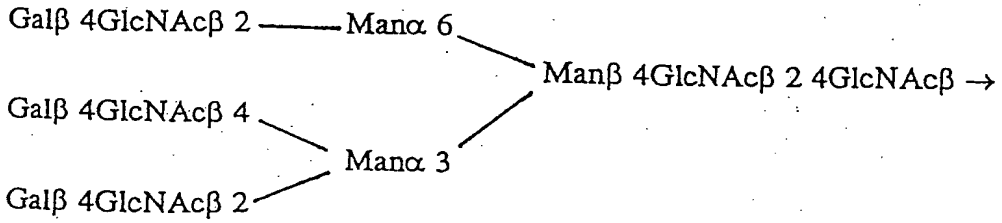
α-MSH: Ac-SYSMEHFRWGKPV-NH_2.

4. The complex of claim 1, wherein :

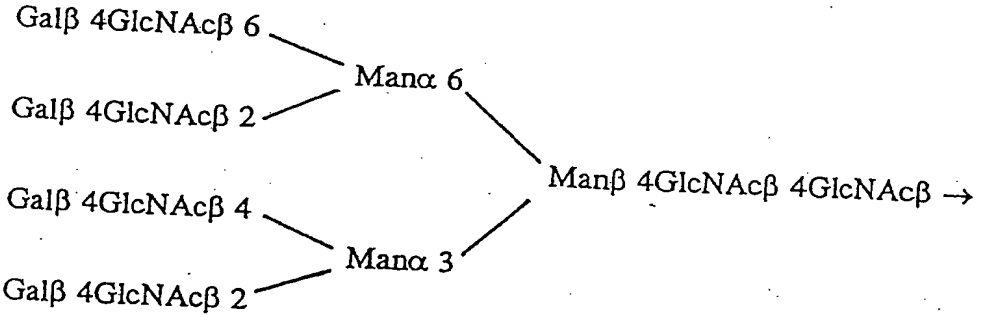
- the monosaccharide is selected from the group consisting of galactose, mannose, fucose, glucose, ribose, xylose and rhamnose,

- the oligosaccharide is chosen from :

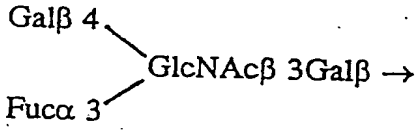
(a) Asialo-oligoside of the type of triantennar lactosamine : asialoglycoprotein receptor



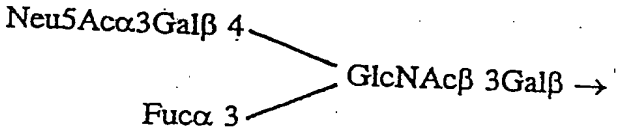
(b) Asialo-oligoside of the type of tetraantennar lactosamine : asialoglycoprotein receptor



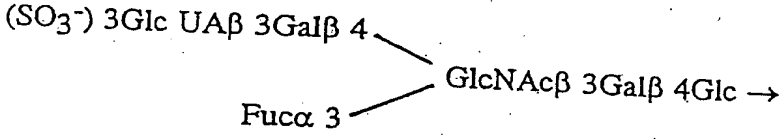
(c) Lewis x : LECAM 2/3



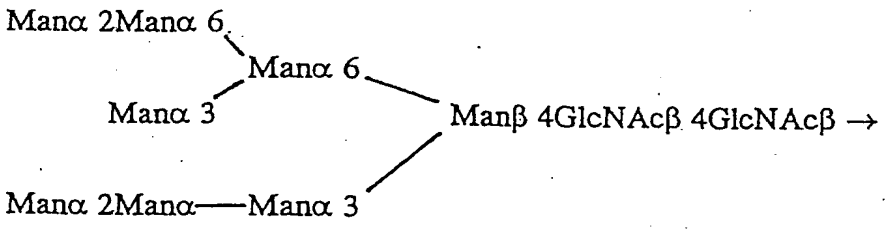
(d) Lewis x sialyl : LECAM 3/2



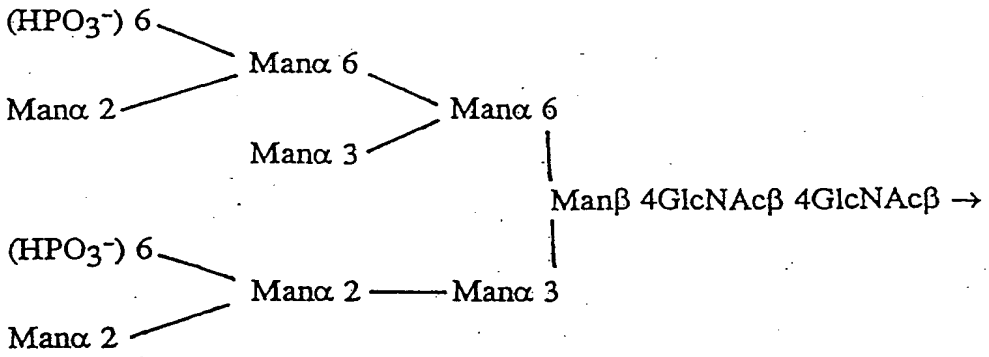
(e) Sulphated Lewis x derivative (HNK1) : LECAM 1



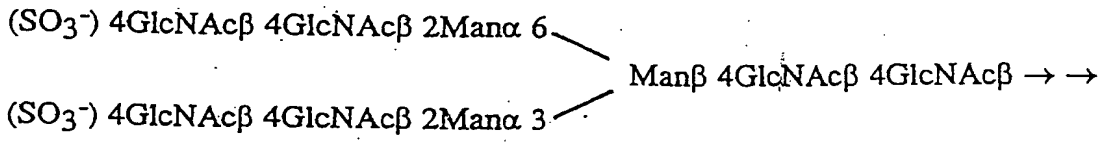
(f) Oligomannoside : mannose receptor



(g) Phosphorylated oligomannoside : mannose 6-phosphate receptor



(h) Oligosaccharide of the type of sulphated lactosamine : sulphated GalNAc 4 receptor :



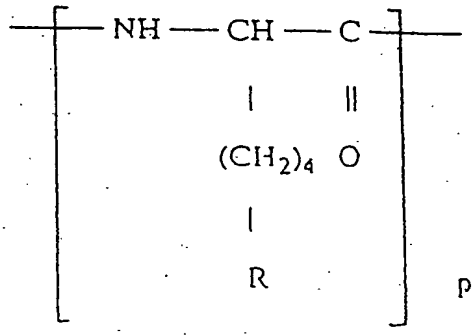
i. Lactose

j. Fucα2Galβ3(Fucα4)GlcNAcβ1Galβ4Glc

k. Fucα4(Galβ3)GlcNAcβ3Galβ

l. Manα6-man

5. The complex of claim 1 wherein the polymeric conjugate has the following formula :



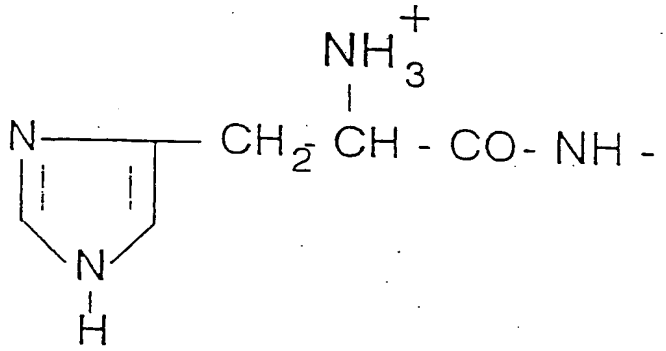
(I)

wherein :

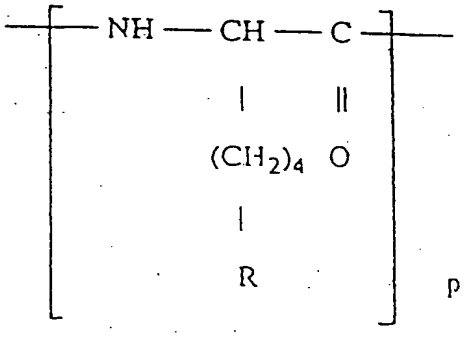
- p is an integer from 15 to 900,
- 10 to 45% of the radical R being a residue with an imidazole nucleus,
- 10 to 90% of R being free NH_3^+ groups,
- and optionally 0 to 45% of R being -NH-CO-$(CHOH)_m$-R_1, m is an integer from 2 to 15 and R_1 is hydrogen or alkyl of 1 to 15 carbon atoms, in particular R being dihydroxypropionylamido, erythronylamido, threonylamido, ribonylamido, arabinylamido,

xylonylamido, lyxonylamido, gluconylamido, galactonylamido, mannonylamido, glycoheptonylamido or glycooctonylamido radical.

6. The complex of claim 5 wherein R is a residue with an imidazole nucleus having the formula :

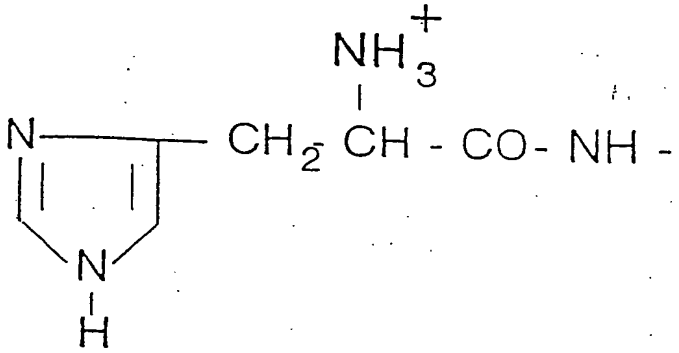


7. The complex of claim 5 wherein the polymeric conjugate has the following formula :



wherein :

- p is an integer from 15 to 900,
- 10% to 45% of R are a residue having an imidazole nucleus and optionally a free NH_3^+ function, in particular a histidyl residue, it being possible for R to be represented by the formula :



- the remainder of the radicals, that is to say 30% to 90% of the number of radicals R, representing free NH_3^+ groups, and it being possible for 0 to 45% of the radicals R to be

substituted by a molecule which constitutes a recognition signal recognized by a cell membrane receptor,

with the proviso that all the free NH_3^+ functions make up at least 30% of the number of monomer units of the polymeric skeleton of the abovementioned polymeric conjugate.

8. A complex according to claim 1 wherein the nucleic acid is selected from the group consisting of :

a) marker genes, such as

- genes containing luciferase,
- green protein of the jellyfish *Aequarea victoria* (« green fluorescent protein » (GFP)),
- genes containing β-galactosidase,
- genes containing chloramphenicol acetyltransferase and,
- genes which confer resistance to an antibiotic, such as hygromycin or neomycin,

b) genes with a therapeutic purpose, such as cystic fibrosis transmembrane conductance regulator (cystic fibrosis (CFTR)),

9. Positively charged polymeric conjugate containing a polylysine formed from monomers having free NH_3^+ groups,

at least 10% of the free NH_3^+ groups of the said polylysine are substituted by residues which can be protonated in a weakly acid medium causing destabilization of cell membranes,

and optionally some of the free NH_3^+ groups of the said polylysine can be substituted by a molecule with a recognition signal recognized by a cell membrane receptor,

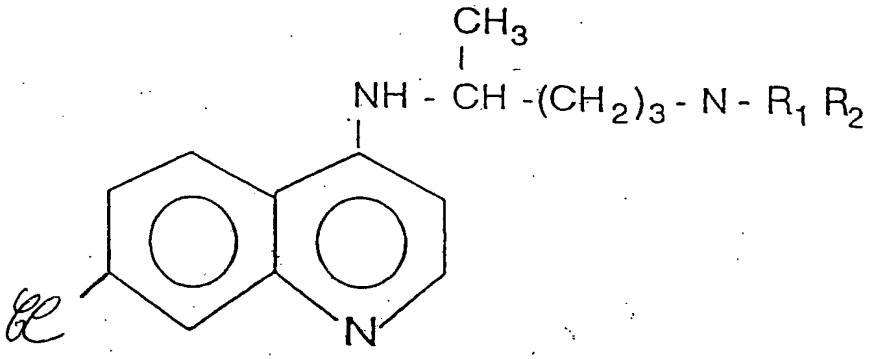
with the proviso that all the free NH_3^+ groups of the said polylysine make up at least 30% of the number of monomers of the skeleton of the polymeric conjugate,

wherein said residues causing destabilization of cell membranes in a weakly acid medium belong :

- to the family of compounds which carry an imidazole nucleus such as alkylimidazoles in which the alkyl radical contains 1 to 10 carbon atoms and in which only one of the nitrogen atoms of the imidazole nucleus is substituted, with said residues causing destabilization of cell membranes being more particularly selected from the group consisting of histidine, 4-carboxymethyl-imidazole, 3-(3-methyl-imidazol-4-yl)-alanine, (3-methyl-

histidine), 3-(1-methyl-imidazol-4-yl)-alanine, (1-methyl-histidine), 2-carboxy-imidazole, histamine, 3-(imidazol-4-yl)-L-lactic acid, 2-(1-methyl-imidazol-4-yl)ethylamine, 2-(3-methyl-imidazol-4-yl)ethylamine, β-alanyl-histidine (carnosine), N-acetyl-histidine,

. to the family of quinolines of the formula :



in which R_1 is hydrogen, R_2 is $-(CH_2)_n-CO_2-H$ and n is an integer from 1 to 10,

with said quinolines being more particularly selected from the group consisting of 7-chloro-4-(amino-1-methylbutylamino)-quinoline, N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine, 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline (primaquine), N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine,

. to the family of pterines such as pteroic acid,

. to the family of pyridine such as nicotinic acid or quinolinic acid,

wherein said recognition signal is selected from the group consisting of :

. simple osides such as a monosaccharide selected from the group consisting of α or β conformers of 2-deoxy, of 2-amino or of 2-deoxy, 2-acetamido neutral monosaccharides ; α or β conformers of glycuronic acid derivatives of neutral monosaccharides ; α or β conformers of L-iduronic acid, of keto-deoxy-octonic acid, of N-acetyl-neuraminic acid, or of N-glycoloyl-neuraminic acid ; and α or β conformers of neutral 6-deoxy monosaccharides ;

or a disaccharide selected from the group consisting of lactose and mannopyranosylα-6mannopyranose,

or complex osides such as an oligosaccharide selected from the group consisting of $Lewis^a$, $Lewis^b$, $Lewis^x$, oligomannosides and oligolactosamines,

. peptides.

10. Positively charged polymeric conjugate according to claim 10, wherein the free NH_3^+ groups of the polylysine are substituted with a non-charged residue causing a reduction in the positive charge of the polymeric conjugate which facilitates salting out of the nucleic acids upon dissociation of the complex, the said non-charged residue being a gluconyl.

11. A composition comprising the complex of claim 1.

12. A method of transfecting cultured cells comprising incubating said cells in the presence of the composition of claim 11 under conditions wherein said composition enters said cells, and the nucleic acid comprised in the complex of said composition is released.

13. The method of claim 12, wherein the cells are selected from the group consisting of

- cells of haematopoietic strains ;
- dendritic cells ;
- liver cells ;
- skeletal muscle cells ;
- skin cells : . fibroblasts,
  . keratinocytes,
  . dendritic cells,
  . melanocytes;
- cells of the vascular walls ;
  . endothelial ;
  . smooth muscle ;
- epithelial cells of the respiratory tract ;
- cells of the central nervous system ;
- cancerous cells ;
- cells of the immune system.

14. A kit comprising :

1) a polymeric conjugate of claim 9 substituted by a residue causing destabilization of cell membranes in a weakly acid medium and optionally carrying a recognition signal being a function of a target cell optionally bonded beforehand to the polymer conjugate,

2) optionally a plasmid containing at least one gene to be transferred and optionally a system for regulation of the expression of the said gene,

3) reagents allowing optional bonding of the recognition signal to the polymeric conjugate,

4) reagents for effecting the formation of a complex of claim 1 or a complex of the polymeric conjugate and the gene to be transferred or between the polymeric conjugate and the plasmid containing the gene to be transferred and,

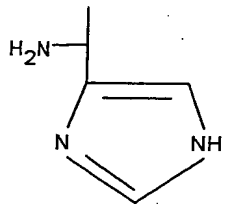
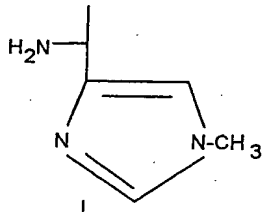
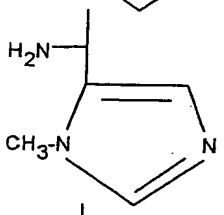
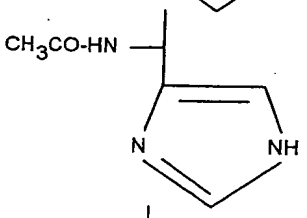
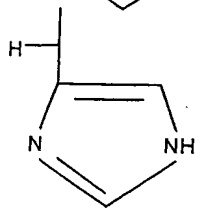
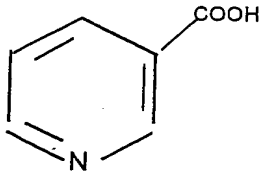
5) reagents for transfection of the cell by the complex of claim 1.

II) Experimental results added by the Applicant of the present invention in order to support the new set of claims and to overcome the Examiner's objections.

The experimental results below comprise A) a Table 1 and B) Figures 10 to 18 and their description.

A) TABLE 1

The **Table 1** below represents the polyfection efficiency of polylysine substituted with different residues causing destabilization of cell membrane in weakly acidic medium.

pLK substitution	residue	% of transfection
	histidyl	100
	1-methyl-histidyl	57
	3-methyl-histidyl	31
	N-acetyl-histidyl	77
	4-carboxymethyl-imidazol	172
	nicotinyl	83

HepG2 cells were transfected with pCMVLuc complexed with various polylysine conjugates. After 48 h, the gene expression was measured by analysis of the luciferase activity in cell lysates. The transfection efficiency is expressed as the percentage relative to the luciferase acitity in cells transfected with polylysine substituted with histidyl residues.

The preparation of polylysine substituted with different residues causing destabilization of cell membrane in weakly acidic medium (see table 1) is described below.

• **Preparation of N-acetyl-histidylated polylysine** (Table 1).

Polylysine *p*-toluene sulfonate salt (50 mg ; 0.87 μmol) in 3 mL of DMSO is reacted in the presence of DIEA (diisopropylethylamine) (60 μL ; 344 μmol) for 24 h at 20°C with *N*-acetyl-histidine (18.8 mg; 0.96 μmol) in the presence of 84.5 mg of BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate ; 1.92 μmol). The polymer is precipitated by adding 10 volumes of isopropanol and collected by centrifugation (1800 g for 15 min). The pellet is washed with isopropanol, collected by centrifugation, solubilized in distilled water and freeze-dried. The average number x of N-acetyl-histidyl residues bound per pLK molecule is determined by ^{1}H NMR spectroscopy at 300 MHz in D_2O according to :

$$x=6(h_{8.7}/h_{lys})DP,$$

wherein $h_{8.7}$ is the value of the integration of the signal at 8.7 ppm corresponding to the proton (C2-H) of *N*-acetyl-histidyl residues, h_{lys} is the value of the integration in the range 1.3-1.9 ppm, corresponding to the 6 methylene protons of lysyl residues, and DP is the degree of polymerization of pLK. The average number of N-acetyl-histidyl residues per pLK molecule is 100.

• **Preparation of 1-methyl-histidylated and 3-methyl-histidylated polylysine** (Table 1).

Polylysine *p*-toluene sulfonate salt (50 mg ; 0.87 μmol) in 3 mL of DMSO is reacted in the presence of DIEA (71 μL ; 407 μmol) for 24 h at 20°C with either 1-methyl-histidine or 3-methyl-histidine (13 mg ; 0.77 μmol) in the presence of 39 mg of BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate ; 0.9 μmol). The polymer is precipitated by adding 10 volumes of isopropanol and collected by centrifugation (1800 g for 15 min). The pellet is washed with isopropanol, collected by centrifugation, solubilized in distilled water and freeze-dried. The average number of 1-methyl- or 3-methyl-histidyl

residues (x) bound per pLK molecule is determined by ^{1}H NMR spectroscopy at 300 MHz in D_2O according to:

$$x=6(h_{his}/h_{lys})DP,$$

wherein h_{his} is the value of the integration of the signal at 3.3 and 3.2 ppm corresponding to methyl protons of 3-methyl-histidyl and 1-methyl-histidyl residues respectively, h_{lys} is the value of the integration in the range 1.3-1.9 ppm, corresponding to the 6 methylene protons of lysyl residues, and DP is the degree of polymerization of pLK. The average number of 1-methyl- and 3-methyl-histidyl residues per pLK molecule was 75.

• **Preparation of polylysine substituted with 4-carboxymethyl-imidazole residues** (Table 1).

Polylysine *p*-toluene sulfonate salt (100 mg ; 1.75 µmol) in 10 mL of DMSO is reacted in the presence of DIEA (75 µL ; 430 µmol) for 24 h at 20°C with 4-carboxymethyl-imidazole (32 mg ; 2.3 µmol) in the presence of 100 mg of BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate ; 4.1 µmol). The polymer is precipitated by adding 10 volumes of isopropanol and collected by centrifugation (1800 g for 15 min). The pellet is washed with isopropanol, collected by centrifugation, solubilized in distilled water and freeze-dried. The average number of 4-carboxymethyl-imidazole residues (x) bound per pLK molecule is determined by ^{1}H NMR spectroscopy at 300 MHz in D_2O according to:

$$x=6(h_{his}/h_{lys})DP,$$

wherein h_{his} is the value of the integration of the signal at 7.9 ppm corresponding to the proton (C2-H) of the 4-carboxymethyl-imidazole residues, h_{lys} is the value of the integration in the range 1.3-1.9 ppm, corresponding to the 6 methylene protons of lysyl residues, and DP is the degree of polymerization of pLK. The average number of 4-carboxymethyl-imidazole-histidyl residues per pLK molecule is 67.

• **Preparation of polylysine substituted with nicotinyl residues** (Table 1).

Polylysine *p*-toluene sulfonate salt (50 mg ; 0.88 µmol) in 4 mL of DMSO is reacted in the presence of DIEA (38 µL ; 217 µmol) for 24 h at 20°C with nicotinic acid (8 mg ; 0.65 µmol) in the presence of 36 mg of BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate ; 1.71 µmol). The polymer is precipitated by adding 10 volumes of isopropanol and collected by centrifugation (1800 g for 15 min). The pellet is washed with isopropanol, collected by centrifugation, solubilized in distilled water and freeze-dried. The average number of nicotinyl residues per pLK molecule is 67.

• **Conclusion:**

Polyfection efficiency of polylysine substituted with different residues causing destabilization of cell membrane in weakly acidic medium (Table 1).

Although, polyplexes made with pLK substituted with 4-carboxymethyl-imidazolyl residues are about 2 fold more efficient than those made with His-pK, the substitution of pLK with histidyl residues is preferred (Table 1). Indeed, 4-carboxymethyl-imidazole substitution often gives polymers with a uncontrolled brown color occurring during the synthesis. Methyl substitution of the nitrogen atoms of the imidazole ring gives less efficient vectors and acetylation of the α-amino group of His decreases slightly the transfection efficiency (Table 1).

B) Description of Figures 10 to 18

(see figures 10 to 18 herewith enclosed)

• **Figure 10** :

This shows the pH-dependence membrane permeabilization induced by histidylated polylysine. The permeabilization of the plasma membrane of non-adherent cells can be easily measured by flow cytometry using ethidium bromide as previously described (Midoux et al., 1995 BBA **1239**, 249-256). This non-fluorescent and poorly membrane-permeant molecule enters the cells upon membrane permeabilization and becomes strongly fluorescent upon binding to DNA. KG1 cells (5 x 10^5 cells) in 1 ml of sheath fluid (134 mM NaCl, 3.7 mM KCl, 15.2 mM NaF, 1.9 mM KH_2PO_4, 16.5 mM Na_2HPO_4, 0.2% 2-phenoxyethanol, pH 7.4) are incubated at 20°C with 12.5 μM BET in the absence (■) or in the presence of either (•) 36 μM peptide H5WYG (GLFHAIAHFIHGGWHGLIHGWYG) (described in Midoux et al., 1998 Bioconjugate Chem. **9**, 260-267), (O) 36 μM peptide E5CA (GLFGAIAGFIEGGWTGMIDGWYG) (described in Midoux et al., 1993 NAR **21**, 871-878) or (▲) 20 μM His-pLK. The pH of the cell suspension is lowered by addition of aliquots of a 0.3N HCl solution. After 15 min, the red cell fluorescence intensity is measured by flow cytometry (λexc. = 488 nm ; λem = 585 ± 44 nm) and the percentage of red labeled cells is determined. In the presence of 36 μM H5WYG or 20 μM His-pLK, 97% of the cells are labeled at pH 6.2 within 15 min, 50% at pH 6.8 and less than 5% at pH 7.1. H5WYG and His-pLK have a better permeabilizing capacity in slightly acidic medium than the anionic peptide E5CA. Fifty percent of the cells are permeabilized at pH 6.0 with E5CA and no one at pH 6.8.

• Preparation of galactosylated and histidylated polylysine (Figure 11).

Galactosylated and histidylated polylysine (His-,Gal-pLK) was prepared as followed : briefly polylysine *p*-toluene sulfonate salt substituted with 38 β-D-galactosyl residues (20 mg ; 0.30 µmol) (prepared as described in Erbacher et al., 1995 Bioconjugate Chem. 6, 401-410) in 2 ml of DMSO was reacted with the N-hydroxysuccinimidyl derivative of BocHis(Boc)-OH (21µmol) (Bachem) in the presence of DIEA (10 µl ; 57.4 µmol). The polymer was precipitated in isopropanol and the Boc protecting groups were removed in dichloromethane/TFA (80:20 ; v:v) mixture for 6h at 20°C. Dichloromethane and TFA were removed under reduced pressure. His-,Lac-pLK was precipitated in 10 volumes of isopropanol, collected by centrifugation (1800 g for 15 min), washed with isopropanol, solubilized in distilled water, and freeze-dried. The average number of histidyl residues bound per polymer molecule determined by ^{1}H NMR spectroscopy at 300 MHz in D_2O was found to be 60.

• Preparation of lactosylated and histidylated polylysine (Figure 12).

Lactosylated and histidylated polylysine (His-,Lac-pLK) are prepared as followed : briefly polylysine *p*-toluene sulfonate salt substituted with 14 β-D-lactosyl residues (20 mg ; 0.30 µmol) (prepared as described in Midoux et al., 1993 NAR 21, 871-878) in 2 ml of DMSO is reacted with the N-hydroxysuccinimidyl derivative of BocHis(Boc)-OH (21µmol) (Bachem) in the presence of DIEA (10 µl ; 57.4 µmol). The polymer is precipitated in isopropanol and the Boc protecting groups are removed in dichloromethane/TFA (80:20 ; v:v) mixture for 6h at 20°C. Dichloromethane and TFA are removed under reduced pressure. His-,Lac-pLK is precipitated in 10 volumes of isopropanol, collected by centrifugation (1800 g for 15 min), washed with isopropanol, solubilized in distilled water, and freeze-dried. The average number of histidyl residues bound per polymer molecule determined by ^{1}H NMR spectroscopy at 300 MHz in D_2O is found to be 60.

• Preparation of Lewis$^{a/x}$-, His-pLK (Figure 13).

Lewis$^{a/x}$-pGluβAla-OH (8.1 mg ; 6.44 µmol) (prepared as described in Monsigny et al., US Patent 5 595 897) in 0.5 ml DMSO in the presence of DIEA (1.4 µl ; 6.44 µmol) is reacted for 30 min at 20°C with BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate ; 3.8 mg ; 7.1 µmol). Then, polylysine substituted with 109 histidyl residues (10 mg ; 0.175 µmol) in 0.5 ml DMSO in the presence of 16 µl (74

µmol) is added dropwise in the oligosaccharide solution. After 30 min, the polymer is precipitated by adding 10 volumes of isopropanol and collected by centrifugation (1800 g for 15 min). The pellet is washed with isopropanol, collected by centrifugation, solubilized in distilled water and freeze-dried. The average number of Lewis$^{a/x}$ residues per pLK molecule is determined by ^{1}H NMR spectroscopy at 300 MHz in D_2O and found to be 30.

• **Preparation of diMan-, His-pLK (Figure 14).**

Manα-6-Man-pGluβAla-OH (6.8 mg ; 13 µmol) (prepared as described in Monsigny et al., US Patent 5 595 897) in 0.25 ml DMSO in the presence of DIEA (2.3 µl ; 10.6 µmol) is reacted for 30 min at 20°C with BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate ; 6.3 mg ; 11.8 µmol). Then, polylysine substituted with 109 histidyl residues (10 mg ; 0.175 µmol) in 0.75 ml DMSO in the presence of 16 µl (74 µmol) is added dropwise in the oligosaccharide solution. After 30 min, the polymer is precipitated by adding 10 volumes of isopropanol and collected by centrifugation (1800 g for 15 min). The pellet is washed with isopropanol, collected by centrifugation, solubilized in distilled water and freeze-dried. The average number of Manα-6-Man residues per pLK molecule is determined by ^{1}H NMR spectroscopy at 300 MHz in D_2O and found to be 23.

• **Preparation of lac-, His-pLK (Figure 15).**

LacpGluβAla-OH (6.8 mg ; 13 µmol) (prepared as described in Monsigny et al., US Patent 5 595 897) in 0.25 ml DMSO in the presence of DIEA (2.3 µl ; 10.6 µmol) is reacted for 30 min at 20°C with BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate ; 6.3 mg ; 11.8 µmol). Then, polylysine substituted with 109 histidyl residues (10 mg ; 0.175 µmol) in 0.75 ml DMSO in the presence of 16 µl (74 µmol) is added dropwise in the oligosaccharide solution. After 30 min, the polymer is precipitated by adding 10 volumes of isopropanol and collected by centrifugation (1800 g for 15 min). The pellet is washed with isopropanol, collected by centrifugation, solubilized in distilled water and freeze-dried. The average number of Lactosyl residues per pLK molecule is determined by ^{1}H NMR spectroscopy at 300 MHz in D_2O and found to be 20.

• **Transfection of cystic fibrosis cells (Figure 16).**

ΣCFTE cells are incubated for 4 h at 37°C with 5 µg plasmid encoding luciferase under the control of the CMV promoter (pUT650) complexed with either lac-, His-pLK, Lac-

pLK (Midoux et al, 1993 NAR 21, 871-878) or pLK in the presence of 10% FCS. The luciferase activity is measured after 48 h culture and expressed as the relative light units (RLU) per 10^6 cells.

- **Transfection of cystic fibrosis cells (Figure 17).**

This shows the transfer of CFTR gene into a human cystic fibrosis airway epithelial cell line. ΣCFTE cells are transfected for 4 h with 10 µg pCMVCFTR plasmid encoding the CFTR protein under the control of the human cytomegalovirus promoter complexed with 30 µg His-pLK. 24h after the transfection, cells are fixed for 20 min at 20°C with 3% paraformaldehyde in PBS, air dried and stored at –80°C until need. Indirect immunofluorescence is performed to evidence the CFTR protein in transfected cells. The mouse anti-CFTR antibody mAb24-1 (Genzyme Corporation, Cambridge, MA) raised against the amino acid sequence 1377-1480 of the C-terminal domain of the CFTR protein is used at a 1 :100 dilution. Secondary antibodies, goat biotinylated anti-mouse IgG (Boehringer Mannheim France SA, Meylan, France) and fluorescein-labeled streptavidin (Amersham, UK) are used at 1 :100 dilution. The sections are mounted in Vectashield-DAPI solution and examined with an MRC-1024 Bio-Rad confocal inverted microscope. CFTR labeling of (**A**) the plasma membrane (arrow), (**B**) the cytoplasm and (**C**) the perinuclear region (Bar = 10 µm).

- **Transfection of Macrophages (Figure 18).**

This shows the transfer of gene into human macrophages. Human macrophages (huMΦ) possess a mannose receptor and take up mannosylated macromolecules. HuMΦ do not express receptor for lactose and Lewis$^{a/x}$. HuMΦ are obtained by ex-vivo differentiation of PBMC (peripheral blood mononuclear cells) from healty donors cultivated 12 days in the presence of GM-CSF. Macrophages are transfected for 4 h with a mixture of two plasmids (6 µg pLTREGFP + 4 µg pLTRTAT) complexed with 30 µg of either diMan-His-pLK, lac-His-pLK (Figure 14) or Lewis$^{a/x}$-His-pLK complexes. pLTREGFP encodes the green fluorescent protein and pLTRTAT encodes the Tat protein both under the control of the LTR promoter of HIV. Twenty four hours later, 40% of macrophages expressed the GFP protein as evidenced by measuring the cell fluorescence intensity by flow cytometry. Therefore, diMan-His-pLk and probably oligomannosylated histidylated pLK could be attractive synthetic vectors to prepare genetically modified macrophages for immunocellular therapy.

III) Inventive step of the present invention

It is right that D3 has suggested that the protonation of the histidine residues of the viral protein with an acidic pH would be an alternative fusion means, but the Applicant shows in figure 10 herewith enclosed that the incorporation of histine residues to some of the free ε-amino groups of the polylysine allows the permeabilization of the plasma membrane of mammalian cells in a weakly acidic medium. This was not described in D3. Document D3 had shown that polyhistidine destabilized phosphatidylserine liposomes. It was not obvious that it would be efficient on the membrane of living cells.

One of the unexpected results of the present invention is that the polylysine bearing histidine residues is able to destabilize cell membranes in acidic medium when it is complexed with a nucleic acid. Indeed, protonated histidines would be expected to interact with the phosphate groups of the nucleic acid as described for the copolymer Poly(Lys,His) in the copolymer/DNA complexes (Santella, R. M., and Li, H. J. 1997 Interaction between Poly(L-lysine[48], L-histidine[52]) and DNA. *Biopolymers* 16, 1879-1894), and therefore they were expected not to be able to interact with cell membranes in order to induce their destabilization.

Document D3 relates to histidine polymer which is a linear polymer.
In the present invention, histidine is grafted on a linear polymer of polylysine. In this structure, the protonated imidazole rings of the histidine groups which do not interact with the nucleic acid, because of the branched structure of histidylated polymer are able to react with the cellular membrane.

The complexes between DNA and the histidylated polylysine have a global surface charge which is slightly positive (+ 15 mV at neutral pH) (zeta potential). This zeta potential increases up to 40 mV when the pH is lowered, to pH 5.5 due to imidazole protonation. This evidences that the imidazoles do not interact with the nucleic acid (Bello-Roufai and Midoux, 2001, Bioconjugate Chem. 12, 92-99).

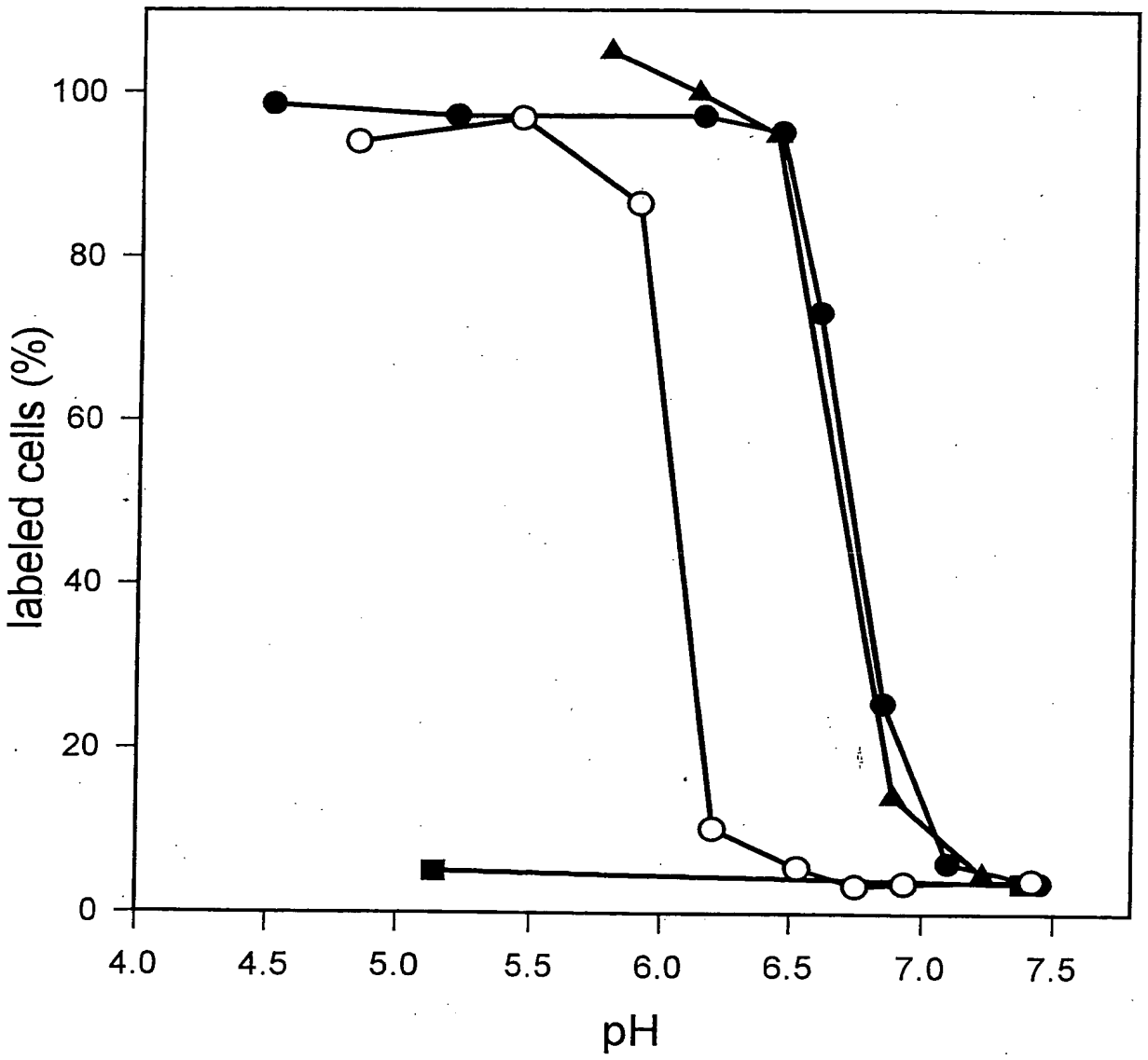
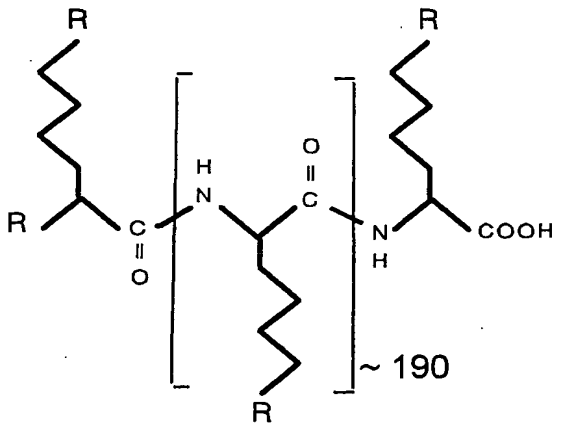
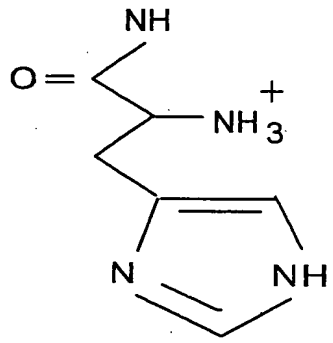


FIGURE 10

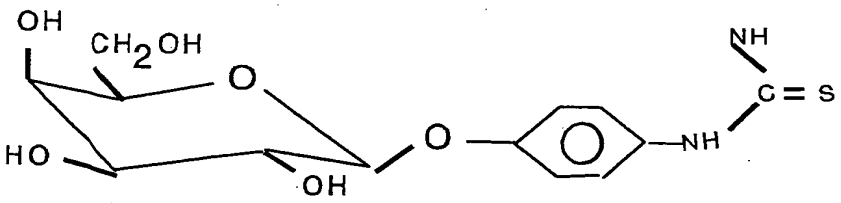


with R =

NH_3^+ (~48%)



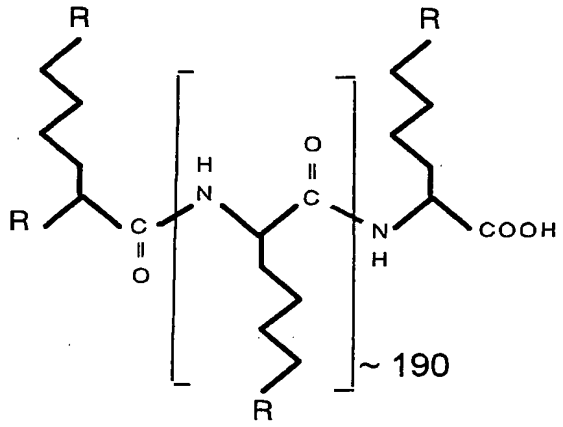
histidylamido (~ 32%)



galactosylβ-phenylthioureido (~20%)

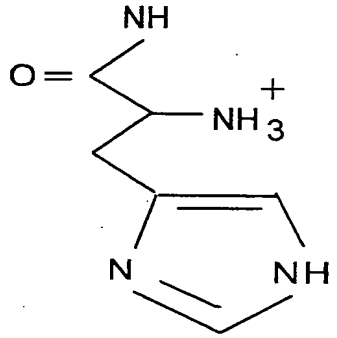
Gal-,His-pLK

Figure 11

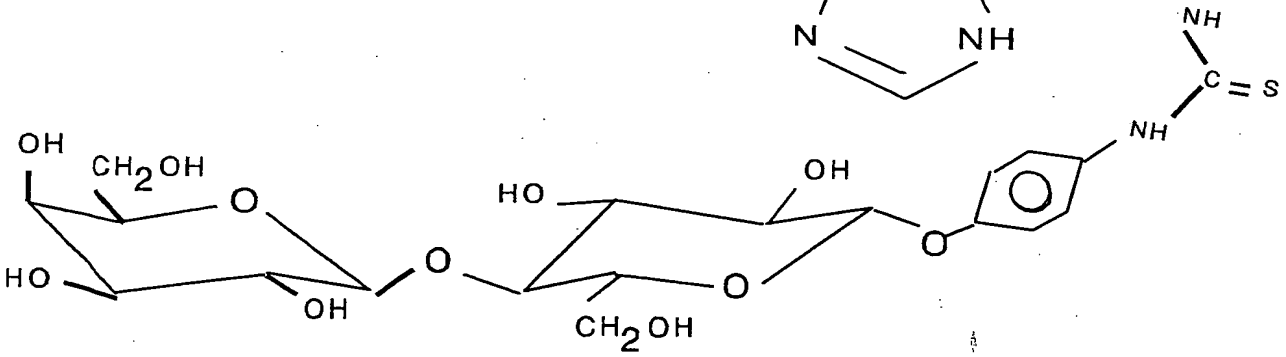


with R =

NH_3^+ (~60%)



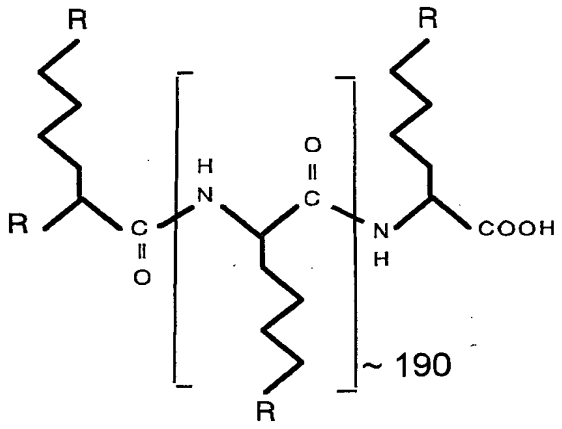
histidylamido (~ 32%)



lactosylβ-phenylthioureido (~8%)

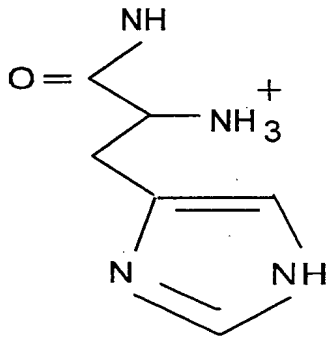
Lac-,His-pLK

FIGURE 12



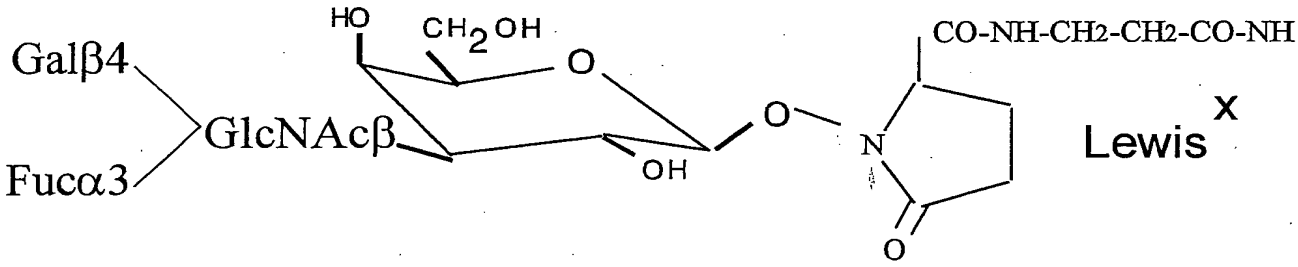
with R =

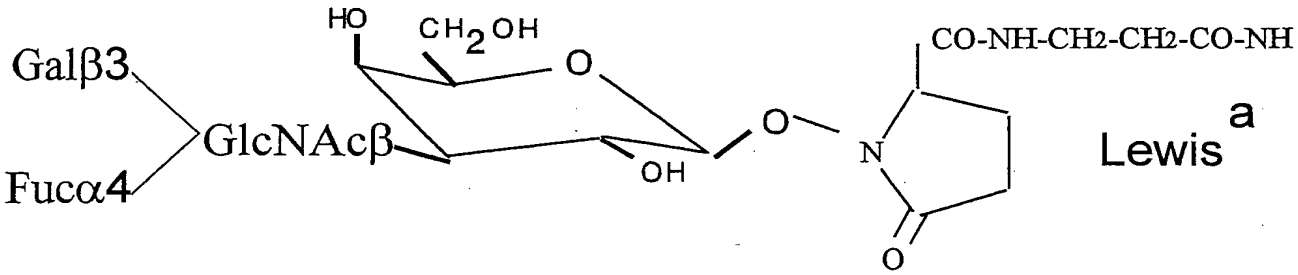
NH_3^+ (~31%)



histidylamido (~ 57%)

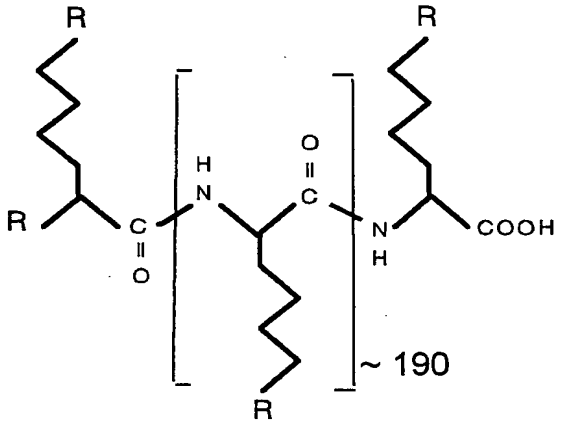
Lewis$^{a/x}$ (~ 12%)





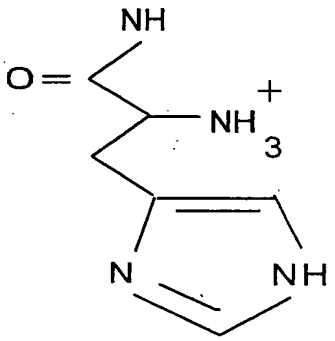
Lewis$^{a/x}$-,His-pLK

FIGURE 13

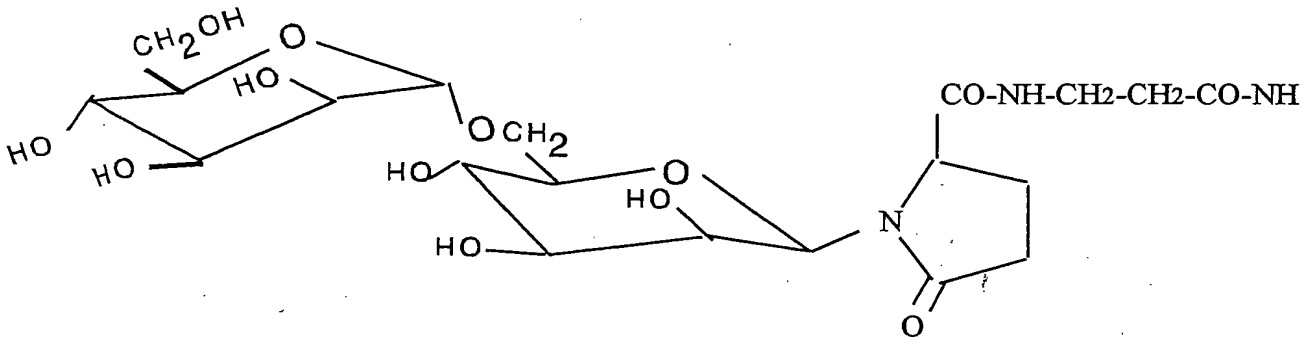


with R =

NH_3^+ (~21%)



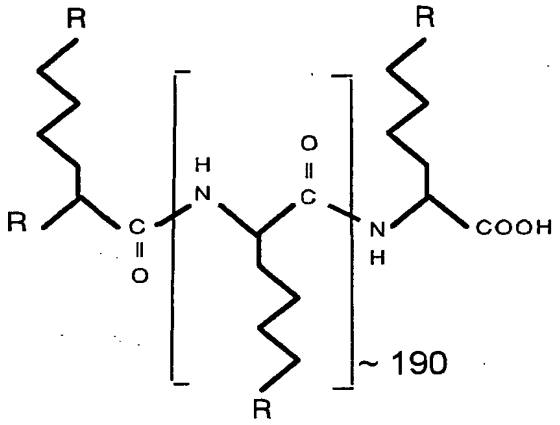
histidylamido (~ 57%)



mannopyranosylα–6mannopyranosylβ
-pyroglutamyl β-alanylamido (~12%)

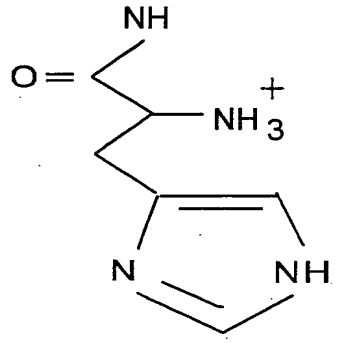
Manα–6Man-,His-pLK

FIGURE 14

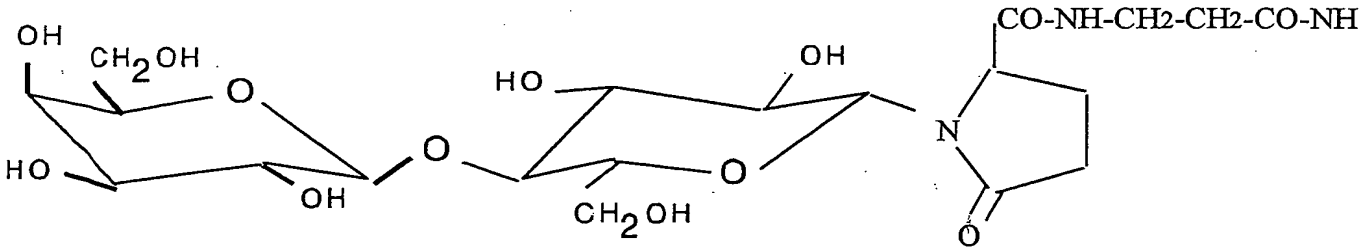


with R =

NH_3^+ (~31%)



histidylamido (~ 57%)



lactosylβ-pyroglutamyl β-alanylamido (~12%)

Lac-,His-pLK

FIGURE 15

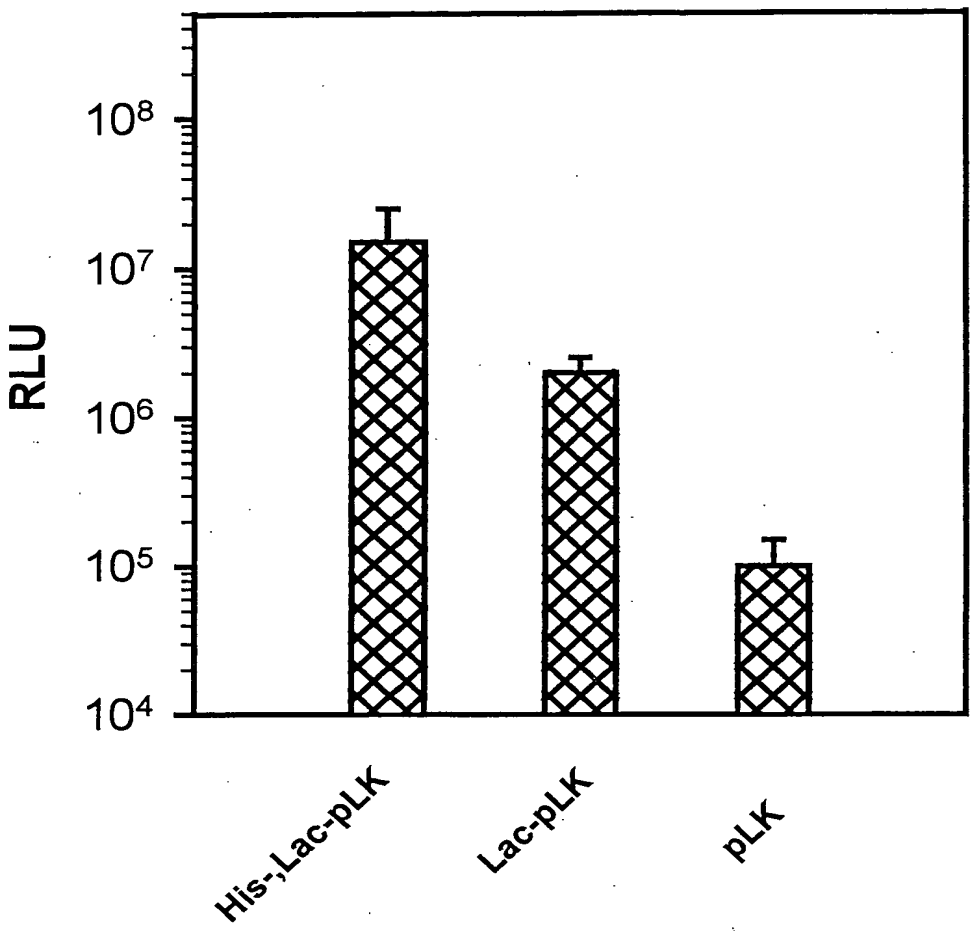


FIGURE 16

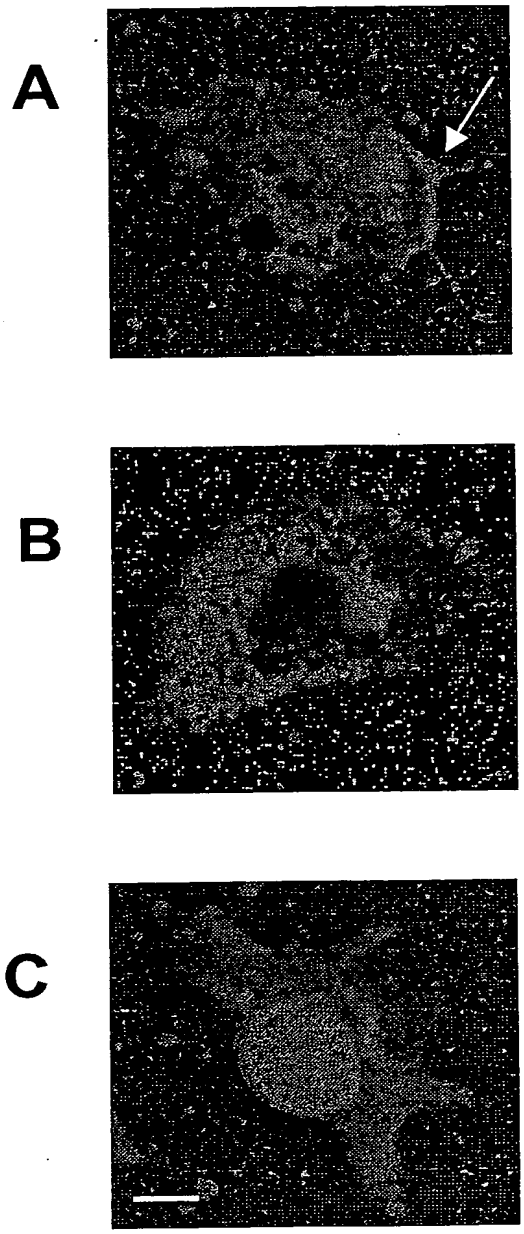
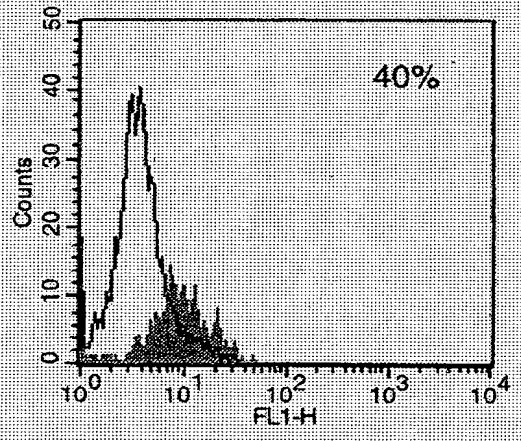


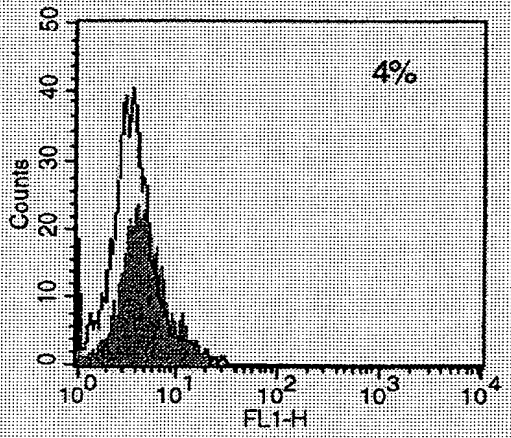
FIGURE 17

Glycofection of adherent huMΦ (monocytes + GM-CSF 12 days)

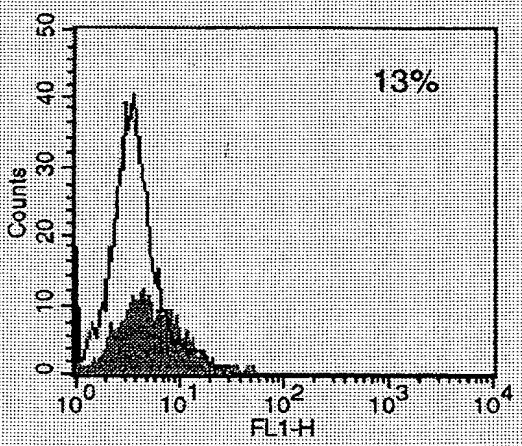
His-pLK-Manα 6Man/ pLTR EGFP+LTR tat



His-pLK-Lac pLTR EGFP+LTR tat



His-pLK-LewisA/X pLTR EGFP+LTR tat



] FIGURE 18

Grosset-Fournier & Demachy

Propriété Industrielle

Grosset-Fournier & Demachy
Membre du G.I.E. PA & GD

Charles Demachy
Docteur en Pharmacie
diplômé du CEIPI
(brevets, marques, modèles)

Catherine Grosset-Fournier
Ingénieur ENSCP
Mandataire Européen
diplômée du CEIPI
(brevets, marques, modèles)

Isabelle Laemmel
Maîtrise de Chimie
diplômée du CEIPI
(brevets, marques, modèles)

Autres membres du G.I.E. :
Pontet & Allano

Sylvain Allano
Docteur ès Sciences
Professeur des Universités

Bernard Pontet
Ingénieur INSA
Conseil en Brevets d'Invention
Mandataire Européen
diplômé du CEIPI
(brevets, marques, modèles)

BIERMAN MUSERLIAN & LUCAS,
600 Third Avenue
New York, NY 10016
USA
Attention : M. C. Muserlian

FACSIMILE / 6 pages

O/Ref. : USB 96 AP IDM HIS/cgf-il

Paris, March 19, 2001

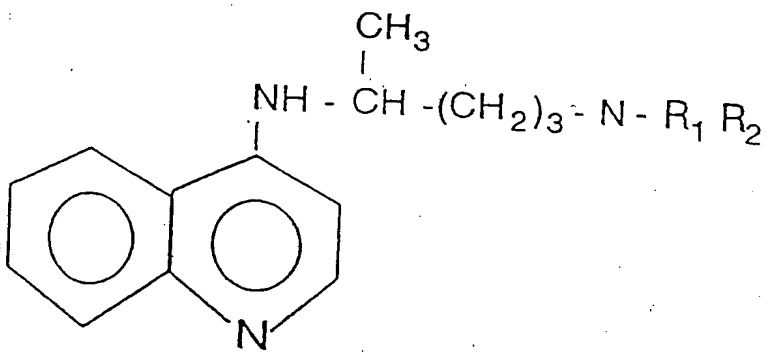
Subject : US Patent Application N°09/297 519 – National phase of the International application n° PCT/FR97/02022 filed on November 10, 1997
Priority : France n°96 13390 filed on November 15, 1996
In the name of : IDM IMMUNO-DESIGNED MOLECULES
Inventors : Midoux et al.
For : « Novel polymeric complexes for the transfection of nucleic acids, with residues causing the destabilisation of cell membranes. »

Dear Chuck,

We refer to our letter of March 15, 2001 concerning the office action of January 18, 2001 received in the above application.

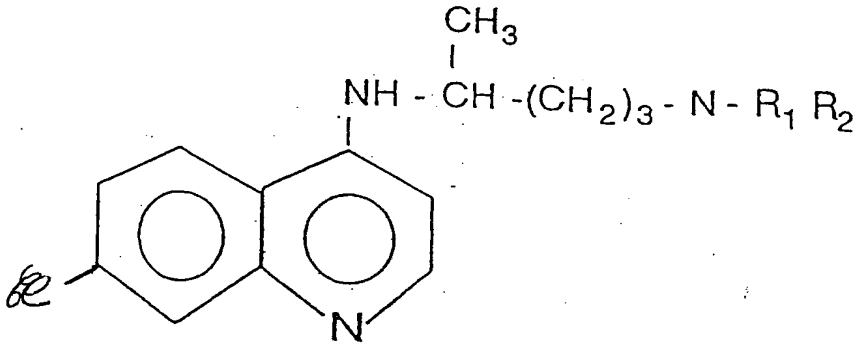
1) a) We draw your attention to the fact that there is an error in our above mentioned letter of March 15, 2001 regarding the formulae of quinolines.

In fact the family of quinolines is represented by the following formula :



(that is to say the family which is represented on page 13 of the present invention).

The compound having the following formula :



is more particularly a chloroquine, and it constitutes a specific example of the above mentioned compound of the quinoline family.

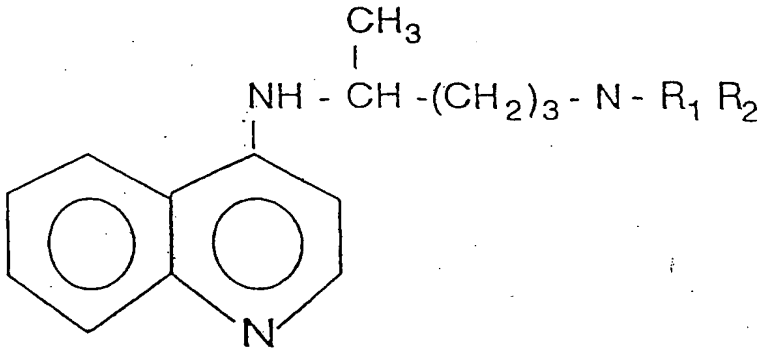
b) Thus we thank you for amending **claims 1 and 9** of the set enclosed with our letter of March 15, 2001 of the following manner :

« ....

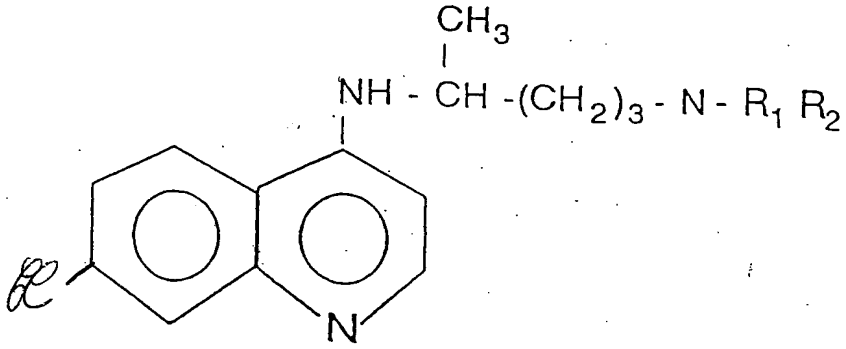
wherein said residues causing destabilization of cell membranes in a weakly acid medium belong :

...

- *to the family of quinolines of the formula :*



in which R_1 is hydrogen, R_2 is $-(CH_2)_n-CO_2-H$ and n is an integer from 1 to 10, and in particular to the family of quinolines of the formula :



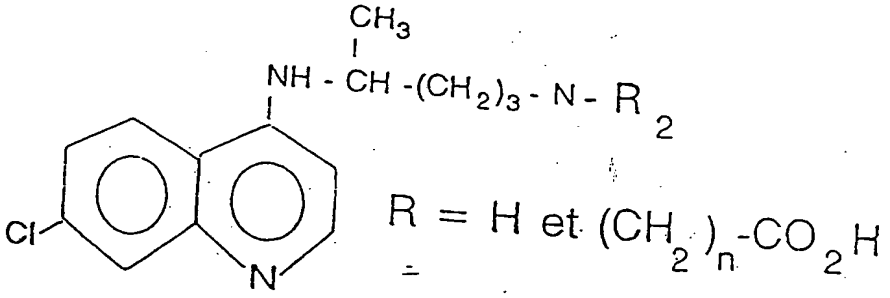
in which R_1 is hydrogen, R_2 is $-(CH_2)_n-CO_2-H$ and n is an integer from 1 to 10,

with said quinolines being more particularly selected from the group consisting of 7-chloro-4-(amino-1-methylbutylamino)-quinoline, N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine, 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline (primaquine), N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine,

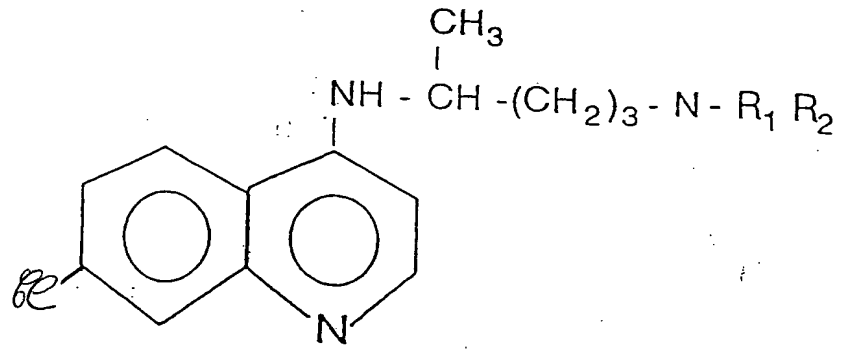
... ».

c) Moreover we draw your attention that the formula of quinoline represented on page 12 of the present invention is erroneous.

Thus, we thank you for replacing the formula of the quinolines represented on page 12 of the present invention :



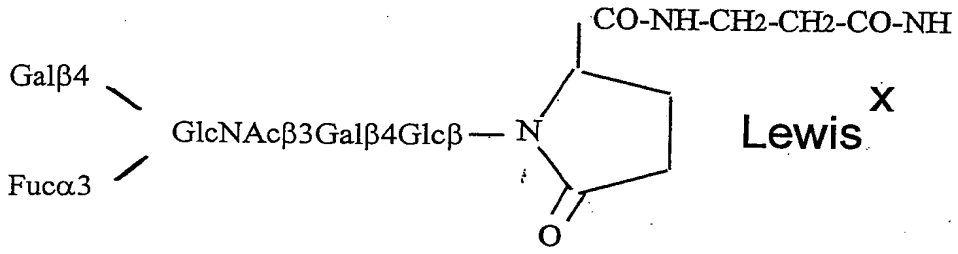
by the following formula :

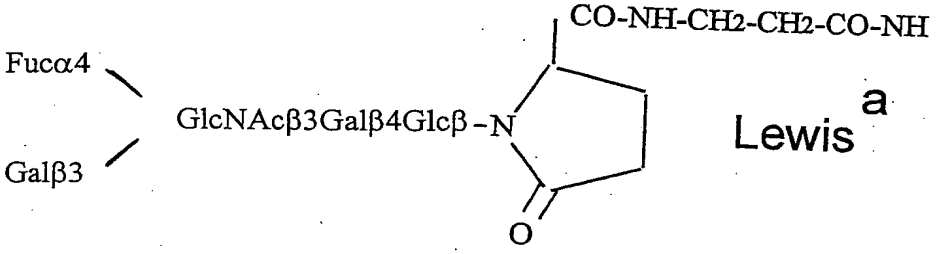


in which R_1 is hydrogen, R_2 is $-(CH_2)_n-CO_2-H$ and n is an integer from 1 to 10.

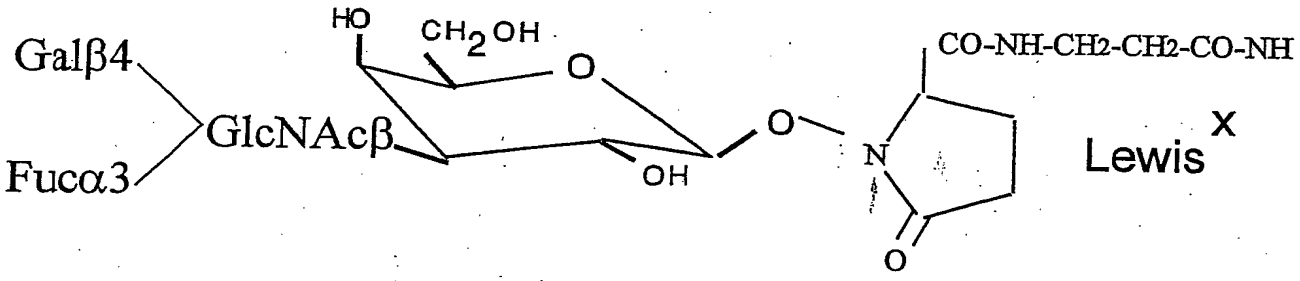
2) We also draw your attention that there is a clerical error on figure 13 concerning the experimental results added by the Applicant in order to overcome the Examiner's objections. In fact there is no oxygene atom between the galactose and the pyroglutamate, and the glucose has been forgotten.

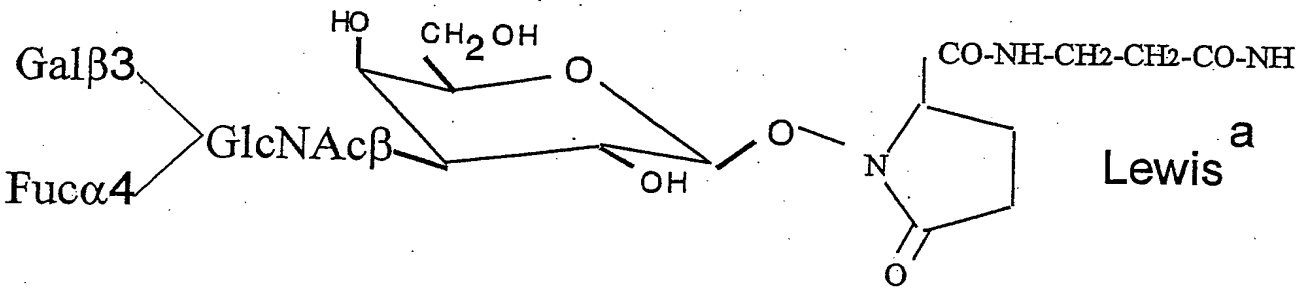
Thus the Lewis^x and Lewis^a compounds are respectively represented by the following formulae :





and not by :





You will find enclosed herewith a new Figure 13.

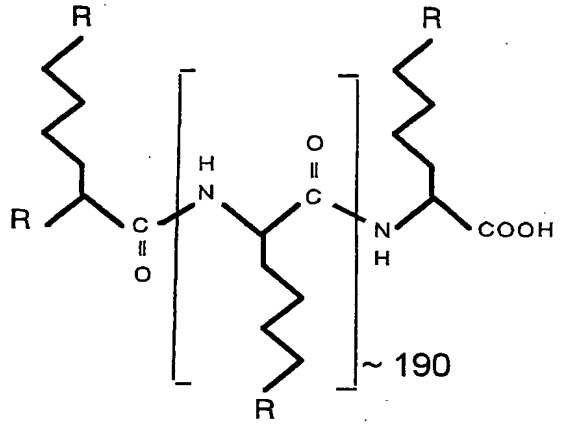
We leave it to you to correct these errors.

We remain at your disposal for any information you may need.

Very truly yours

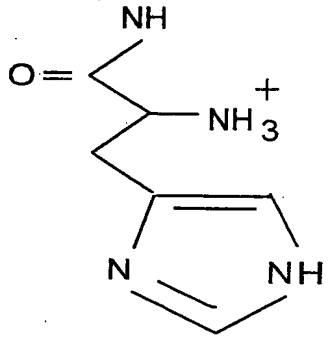
Catherine Grosset-Fournier

Encl : - new figure 13



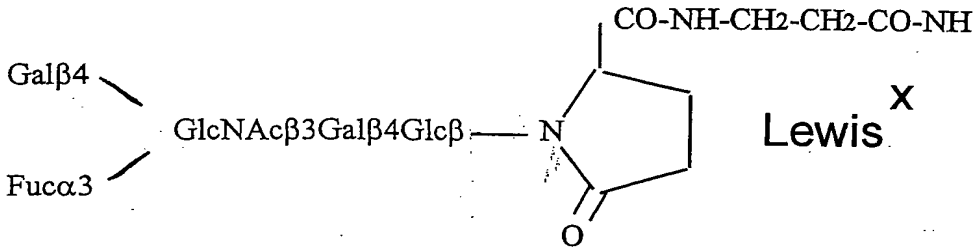
with R =

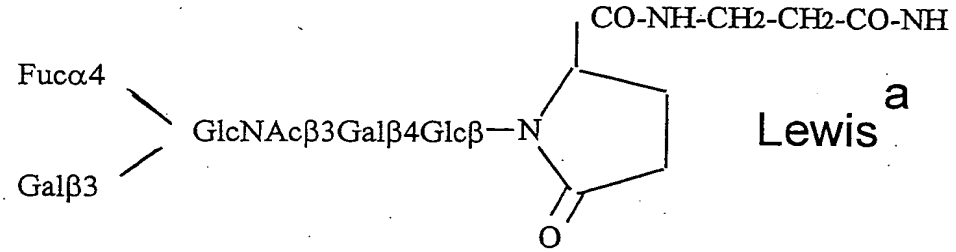
NH_3^+ (~31%)



histidylamido (~ 57%)

Lewis$^{a/x}$ (~ 12%)





Lewis$^{a/x}$-,His-pLK

FIGURE 13

410.015

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: : D. Nguyen
MIDOUX et al :
Serial No.: 297,519 : Group: 1633
Filed: May 3, 1999 :
For: NOVEL POLYMERIC...MEMBRANES :

600 Third Avenue
New York N.Y. 10016
July 17, 2001

AMENDMENT

Asst. Commissioner for Patents
Washington, D.C. 20231

Sir:

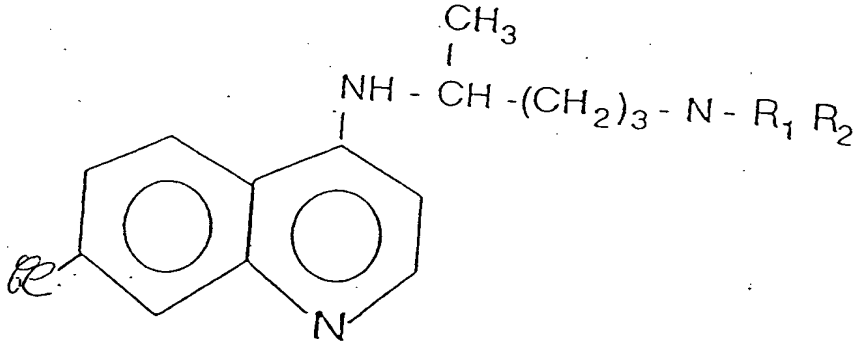
Responsive to the office action of January 18, 2001, please amend this application as follows:

IN THE SPECIFICATION:

Page 1, before line 1, insert

--This application is a 371 of PCT/FR97/02022 filed November 10, 1997.--

Page 12, replace line 12 with the following formula:



IN THE CLAIMS:

Cancel claims 20 to 44 and add the following claims:

--45. A complex comprised of at least one negatively charged nucleic acid and at least one positively charged polymeric conjugate with the bond therebetween being electrostatic in nature,

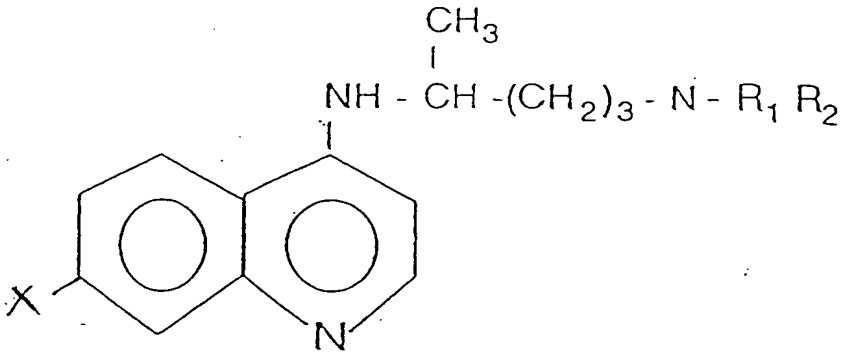
the polymeric conjugate containing a polylysine formed from monomers having free NH_3^+ groups,

at least 10% of free NH_3^+ groups of the said polylysine are substituted by residues which can be protonated in a weakly acid medium causing destabilization of cell membranes,

and optionally some of the free NH_3^+ groups of the said polylysine can be substituted by a molecule with a recognition signal recognized by a cell membrane receptor,

with the proviso that all the free NH_3^+ groups of the said polylysine make up at least 30% of the number of monomers of the skeleton of the polymeric conjugate,

wherein said residue causing destabilization of cell membrane in a weakly acid medium belong to the family of quinolines of the formula:



in which R_1 is hydrogen, R_2 is $-(CH_2)_n-CO_2-H$, X is hydrogen or chlorine and n is an integer from 1 to 10, wherein said recognition

signal is selected from the group consisting of:

simple osides selected from the group consisting of α or ß conformers of 2-deoxy, of 2-amino or 2-deoxy, 2-acetamido neutral monosaccharides; α or ß conformers of glycuronic acid derivatives of neutral monosaccharides; α or ß conformers of L-iduronic acid, of keto-deoxy-octonic acid, of N-acetyl neuraminic acid, or of N-glycoloyl-neuraminic acid; and α or ß conformers of neutral 6-deoxy monosaccharides;

or a disaccharide selected from the group consisting of lactose and mannopyranosylα-6-mannopyranose,

or complex osides selected from the group consisting of Lewis^a, Lewis^b, Lewis^x, oligomannosides and oligolactosamines or peptides.

46. A complex of claim 44 wherein said quinolines are selected from the group consisting of 7-chloro-4-(amino-1-methylbutylamino)-quinoline, N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine, 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline (pyrimaquine), N^4-(6- methoxy-8-quinolinyl)-1,4-pentanediamine,

to the family of pterines

and to the family of pyridine.

47. The complex of claim 45 wherein the free NH_3^+ groups of the polylysine are substituted with a non-charged gluconyl residue causing a reduction i the positive charge of the polymeric conjugate which facilitates salting out of the nucleic acids upon dissociation of the complex.

48. The complex of claim 45 wherein said recognition signal

is a peptide chosen from the group consisting of

(a) anti-inflammatory peptides recognized by receptors of the vascular wall,

(b) ligand peptides of integrins,

(c) chemiotactic factors and

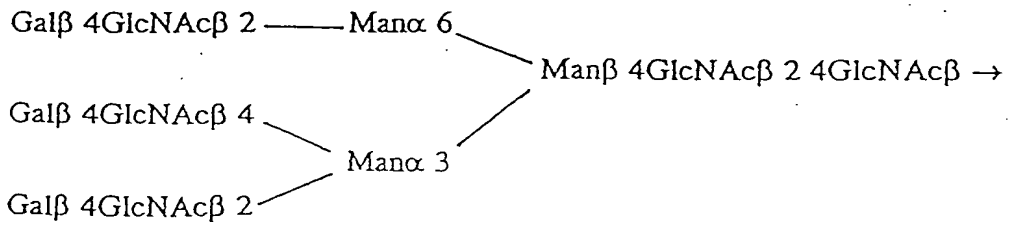
(d) peptide hormones.

49. The complex of claim 45 wherein:

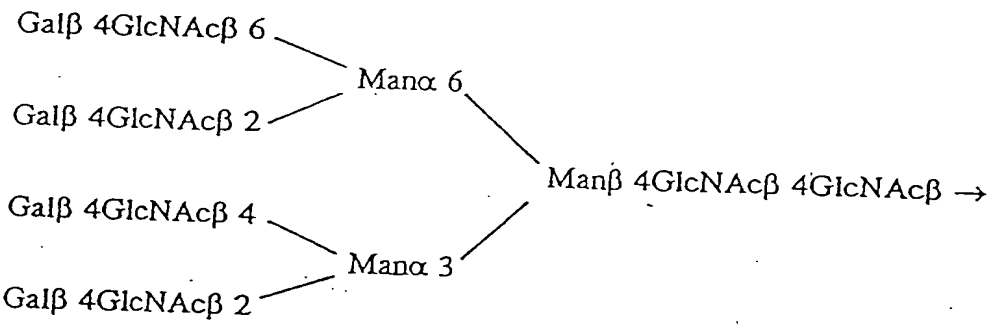
- the monosaccharide is selected from the group consisting of galactose, mannose, fucose, glucose, ribose, xylose and rhamnose and

- the oligosaccharide is selected from the group consisting of

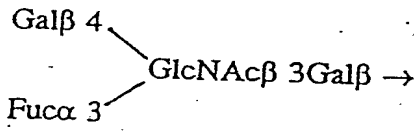
(a) Asialo-oligoside of the type of triantennar lactosamine: asialoglycoprotein receptor



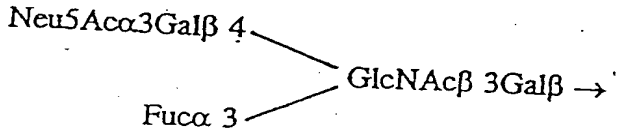
(b) Asialo-oligoside of the type of tetraantennar lactosamine : asialoglycoprotein receptor



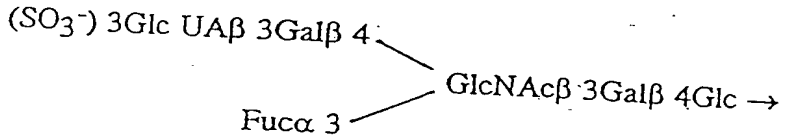
(c) Lewis x : LECAM 2/3



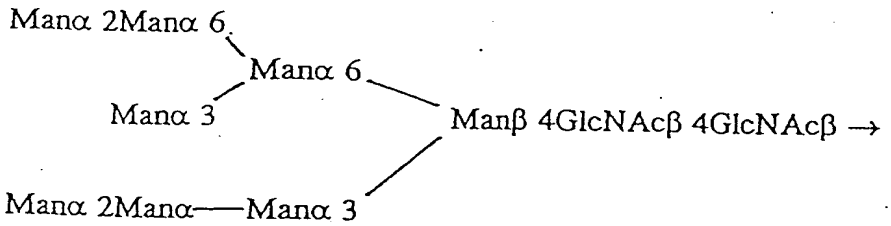
(d) Lewis x sialyl : LECAM 3/2



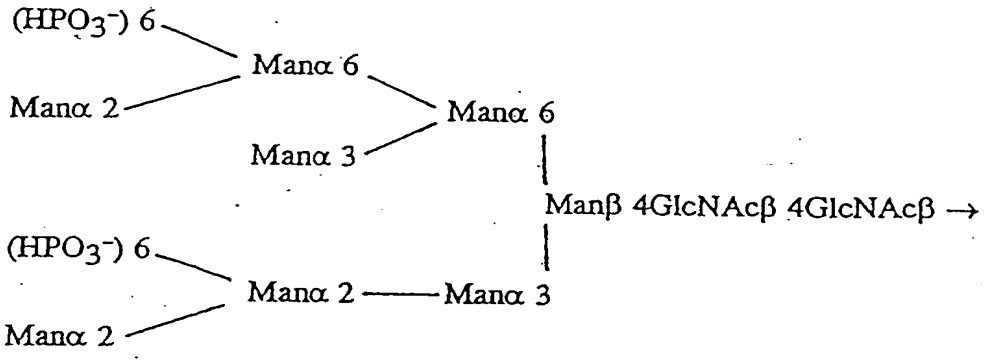
(e) Sulphated Lewis x derivative (HNK1) : LECAM 1



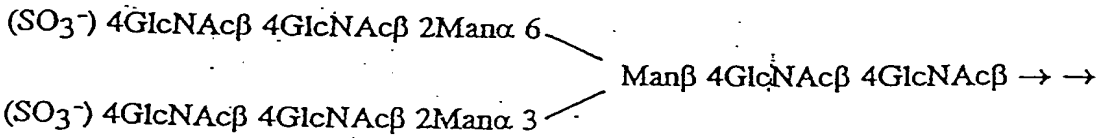
(f) Oligomannoside : mannose receptor



(g) Phosphorylated oligomannoside : mannose 6-phosphate receptor



(h) Oligosaccharide of the type of sulphated lactosamine: sulphated GalNAc 4 receptor:



i. Lactose

j. Fucα2Gakß3(fucα4)GlcNAcß1Galß3Glc

k. Fucα4(Gaß3)GlcNAcß3Galß

l. Manα6-man.

50. The complex of claim 49 wherein the peptides are selected from the group consisting of

- vasodilator intestinal polypeptide (VIP)

HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2

- antrial natriuretic polypeptide (ANP)

SLRRSSCFGGRMDRIGAQSGLGCNSFRY

- lipocortin

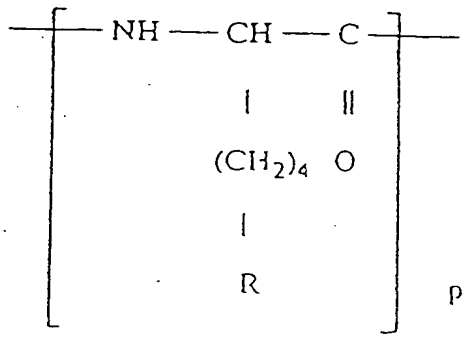
HDMNKVLDL

- bradykinin

RPPGFSPER;

peptides containing the RGC sequence, fibroonectin ligand, formyl peptides and ~~their antagonists, α MSH~~ FMLP, (N-formyl-Met-Leu-Phe) and Ac-SYMEHFRWGKPV-NH_2.

51. The complex of claim 45 wherein the polymeric conjugate has the formula:



I

wherein:

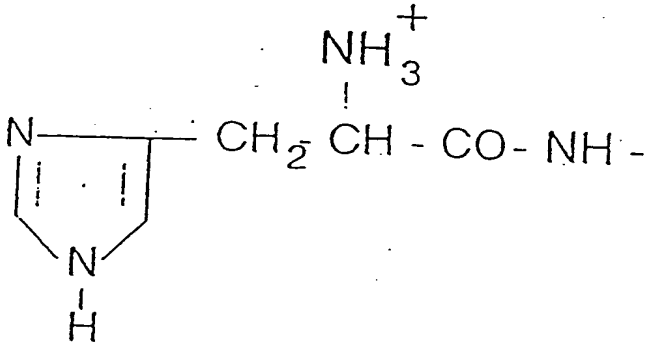
- p is an integer from 15 to 900,

- 10 to 45% of the radical R being a residue with an imidazole nucleus,

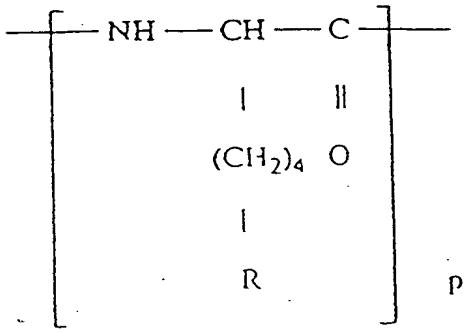
- 10 to 90% of R being free NH_3^+ groups,

- and optionally 0 to 45% of R being -NH-CO-$(CHOH)_m$-R_1, m is an integer from 2 to 15, and R_1 is hydrogen or alkyl of 1 to 15 carbon atoms.

52. The complex of claim 51 wherein R is a residue with an imidazole nucleus of the formula:



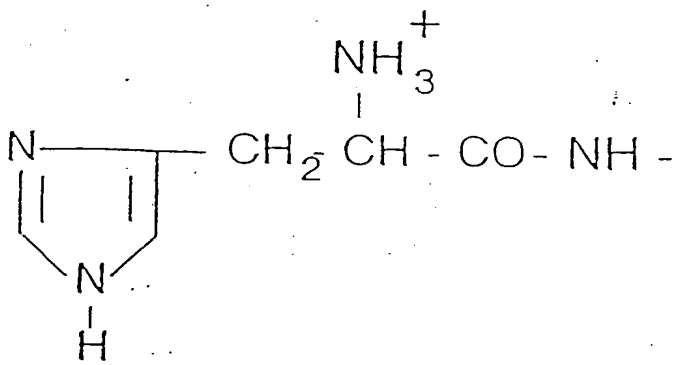
53. The complex of claim 51 wherein the polymeric conjugate has the following formula:

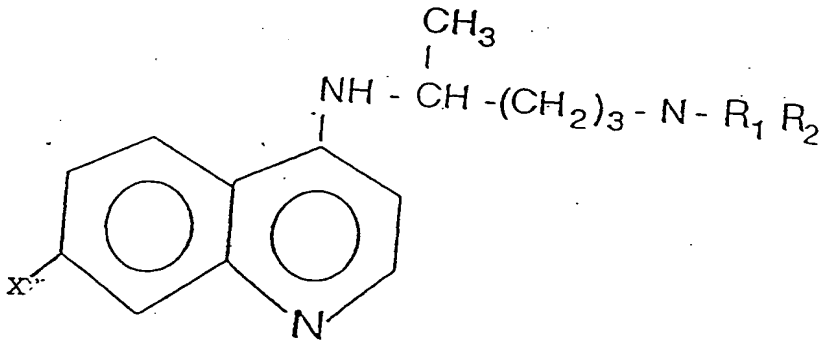


wherein:

- p is an integer from 15 to 900,

- 10% to 45% of R is a residue having an imidazole nucleus and optionally a free NH_3^+, it being possible for R to have the formula:





in which R_1 is hydrogen, R_2 is $(CH_2)_n$-CO_2-H, X is hydrogen or chlorine and n is an integer from 1 to 10, wherein said recognition signal is selected from the group consisting of:

- simple osides selected from the group consisting of α or ß conformers of 2-deoxy, of 2-amino or of 2-deoxy, 2-acetamido neutral monosaccharides; α or ß conformers of glycuronic acid derivatives of neutral monosaccharides; α or ß conformers of L-iduronic acid, of keto-deoxy-octonic acid, of M-acetyl-neuraminic acid, or of N-glycoloyl-neuraminic acid; and α or ß conformers of neutral 6-deoxy monosaccharides;

- a disaccharide selected from the group consisting of lactose and mannopyranosylα-6-mannopyranose,

and complex osides selected from the group consisting of Lewis^a, Lewis^b, Lewis^x, oligomannosides and oligolactosamines, and peptides.

56. Positively charged polymeric conjugate according to claim 55 wherein the free NH_3^+ groups of the polylysine are substituted with a non-charged residue causing a reduction in the positive charge of the polymeric conjugate which facilitates salting out of the nucleic acids upon dissociation of the complex, the said non-

charged residue being a gluconyl.

57. A composition comprising the complex of claim 45 and an inert pharmaceutical carrier.

58. A method of transfecting cultured cells comprising incubating said cells in the presence of a composition of claim 57 under conditions wherein said composition enters said cells, and the nucleic acid comprised in the complex of said composition is released.

59. The method of claim 58 wherein the cells are selected from the group consisting of

-cells of haematopoietic strains;

-dendritic cells;

-liver cells;

-skeletal muscle cells;

-skin cells;

-fibroblasts,

-keratinocytes,

-dendritic cells,

-melanocytes;

-cells of the vascular walls;

endothelial;

smooth muscle;

-epithelial cells of the respiratory tract;

-cells of the central nervous system;

-cancerous cells;

-cells of the immune system.

Applicants are submitting herewith an Abstract of the Disclosure on a separate sheet of paper. Applicants have cancelled non-elected claims 41 to 44 but reserve the right to file a divisional application directed thereto. The specification has been amended to insert reference to the PCT application.

The sequence listing required by the Examiner has already been submitted on May 1, 2001.

With respect to the Examiner's rejection of the claims under 35 USC 112, second paragraph, it is deemed that the new set of claims comply with the statute and are free of the objections noted by the Examiner in the office action. Therefore, withdrawal of these grounds of rejection is requested.

Claims 21 to 40 were rejected under 35 USC 103 as being obvious over the French '316 or the Midoux et al patents taken with the Wang et al reference. The Examiner states that D1 and Midoux, the two primary references, describe a complex of at least one negatively charged nucleic acid and at least one positively charged polymeric conjugate bonded by electrostatic interaction. The Examiner states that the polymeric conjugate contains a polymer of monomeric units with free NH_3^+ groups which may be substituted in a ratio of at least 10% by gluconyl based non-charged residues. The Examiner states that the difference between the primary references and the present invention is that the claimed invention

is directed to histidine residues that are protonable in a weakly acidic medium and have a functional group enabling them to be bound to the polymer while not being recognized by a cell membrane receptor.

The Examiner states that the secondary reference describes the fusion-mediating properties of polyhistidine relative to liposomes and the concept diffusion is caused by the poly-cationic nature of polyhistidine having an acid pH and the combination of the polycation with membrane phospholids that induce phase separation in the dual lipid layer can be seen from the Abstract. The Examiner further states that the secondary reference indicates that fusion mediating behavior associated with polyhistidine having a low pH is more effective than the one associated with calcium ions or polylysine and deems that it would have been obvious to incorporate histidine residues to any of the free NH_3^+ groups of the polylysine of D1 to enhance the fusion and translocation.

Applicants respectfully traverse these grounds of rejection since in the present invention, polylysine is partially substituted by residues which can induce destabilization of membranes in an acid medium and particularly, histidyl residues and is efficient in the absence of auxiliary elements such as chloroquine or fusiogenic peptides. With respect to the French '31C or D1, this patent relates to a complex between the negatively charged nucleic acid and at least one positively charged polymeric conjugate with the

binding between the nucleic acid and the polymeric conjugate being of electrostatic nature. The polymeric conjugate contains a polymer formed of monomer units carrying free NH_3^+ functions such that the free NH_3^+ functions of the above mentioned units are substituted at a rate of at least 10% of non-charged residues being responsible for a diminution of positive charges with respect to the same substituted polymeric conjugates. Moreover, the patent relates to the preparation and use of polylysine substituted by gluconoyl residues and possibly, biligands of small molecular weight for gene transfer. This modified polylysine requires the use of auxiliary elements such as chloroquine or fusiogenic peptides in an acid medium to make easier the transmembrane passage of DNA into cytosol after endocytosis in the acid besicles.

With respect to the D3 reference of Wang et al, Applicants will concede that this suggests the protenation of the histidine residues of the viral protein with an acidic pH as an alternative fusion means. However, Applicants have shown that the incorporation of histidine residues to some of the free e-amino groups of the polylysine allows the permeabilization of the plasma membrane of mammalian cells in weakly acidic medium. This was not described in the D3 document which shows the polyhistidine destabilized phosphatidylserine liposomes. It was not obvious that it would be efficient on the membrane of living cells. One of the unexpected results of Applicants' invention is that the polylysine bearing histidine residues are able to destabilze cell membranes in

acidic medium when it is complexed with a nucleic acid. Moreover, protenated histidines would be expected to interact with the phosphate groups of the nucleic acid such as described for the polymer poly (Lys, His) in the copolymer/DNA complexes as taught by Santella et al, H.J. 1997 entitled Interaction between poly(L-lysine[48], L-histidine[52]) and DNA, Biopolymers, Vol. 16, pp. 1879-1894. Therefore, they would not be expected to interact with cell membranes to induce their destabilization.

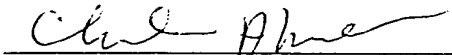
Moreover, the D3 document relates to a histidine polymer which is a linear polymer. In Applicants' invention, histidine is grafted onto a linear polymer of polylysine. In this structure, the protenated imidazole rings of the histidine groups which do not interact with the nucleic acid because of the branch structure of histidylated polymer are able to react with the cellular membrane. the complexes between DNA and histidylated polylysine have a global surface charge which is slightly positive (+15 mV at neutral pH), (state of potential). This state of potential increases up to 40 mV when the pH is lowered to a pH of 5.5 due to imidazole protenation. This means that the imidazoles do not interact with the nucleic acid. Therefore, the combination of the prior art does not anticipate Applicants' invention and withdrawal of this ground of rejection is requested.

Claims 21 to 40 were rejected under the judicially created doctrine of obviousness type double patenting with respect to claims 9 to 15 of U.S. Patent No. 5,733,762 taken alone or in view of the Wang et al reference.

Applicants respectfully traverse this ground of rejection since it is not deemed that the U.S. Patent No. 5,733,762 claims the same invention as this. The said patent corresponds to the D1 reference cited by the Examiner and therefore, the arguments with respect to the lack of teachings of the D1 reference applies to this ground of rejection and therefore, a terminal disclaimer is not required. Therefore, withdrawal of this ground of rejection is requested.

In view of the amendments to the specification and claims and the above remarks, it is believed that the claims clearly point out Applicants' patentable contribution and favorable reconsideration of the application is requested.

Respectfully submitted,
Bierman, Muserlian and Lucas

By: 
Charles A. Muserlian #19,683
Attorney for Applicants
Tel.# (212) 661-8000

CAM:ds
Enclosures

ABSTRACT OF THE DISCLOSURE

A complex comprised of at least one negatively charged nucleic acid and at least one positively charged polymeric conjugate with the bond therebetween being electrostatic in nature,

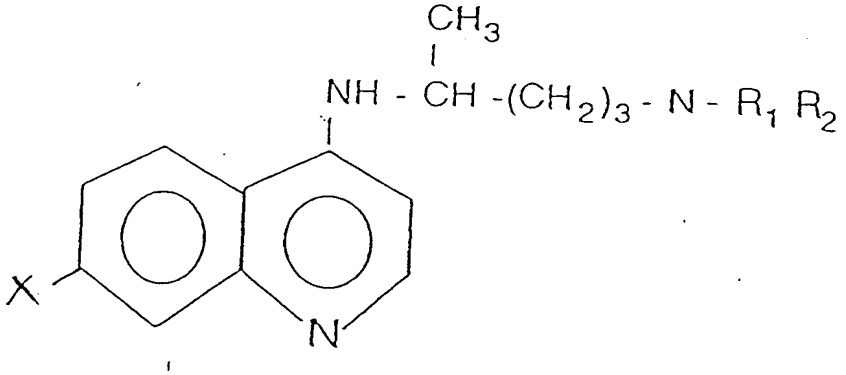
the polymeric conjugate containing a polylysine formed from monomers having free NH_3^+ groups,

at least 10% of free NH_3^+ groups of the said polylysine are substituted by residues which can be protonated in a weakly acid medium causing destabilization of cell membranes,

and optionally some of the free NH_3^+ groups of the said polylysine can be substituted by a molecule with a recognition signal recognized by a cell membrane receptor,

with the proviso that all the free NH_3^+ groups of the said polylysine make up at least 30% of the number of monomers of the skeleton of the polymeric conjugate,

wherein said residue causing destabilization of cell membrane in a weakly acid medium belong to the family of quinolines of the formula:



in which R_1 is hydrogen, R_2 is $-(CH_2)_n-CO_2-H$, X is hydrogen or

chlorine and n is an integer from 1 to 10, wherein said recognition signal is selected from the group consisting of:

simple osides selected from the group consisting of α or ß conformers of 2-deoxy, of 2-amino or 2-deoxy, 2-acetamido neutral monosaccharides; α or ß conformers of glycuronic acid derivatives of neutral monosaccharides; α or ß conformers of L-iduronic acid, of keto-deoxy-octonic acid, of N-acetyl neuraminic acid, or of N-glycoloyl-neuraminic acid; and α or ß conformers of neutral 6-deoxy monosaccharides;

or a disaccharide selected from the group consisting of lactose and mannopyranosylα-6-mannopyranose,

or complex osides selected from the group consisting of Lewis^a, Lewis^b, Lewis^x, oligomannosides and oligolactosamines or peptides.